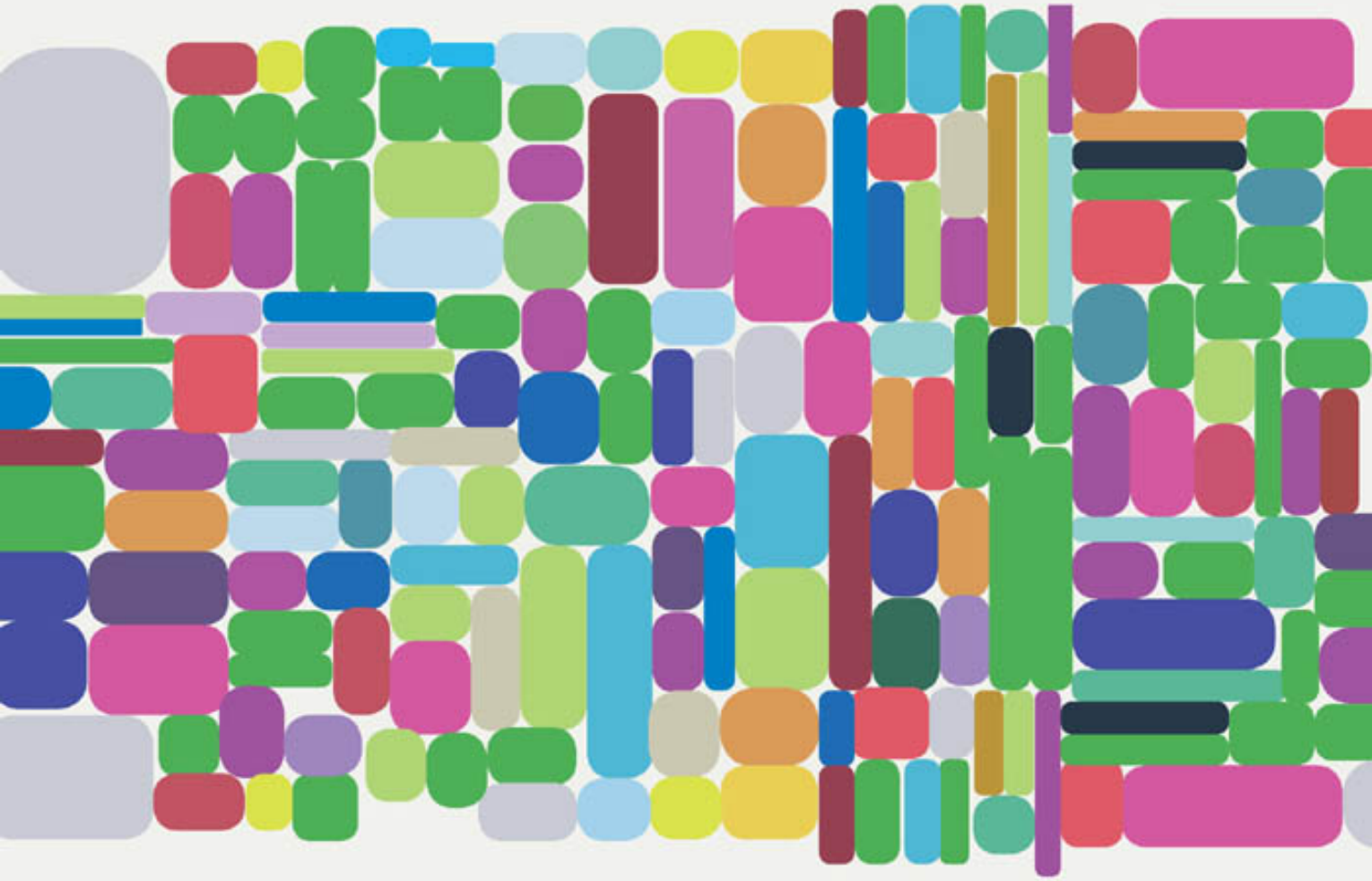
Third Edition

PRIMARY IMMUNODEFICIENCY DISEASES

A Molecular and Genetic Approach



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Hans D. Ochs / C. I. Edvard Smith / Jennifer M. Puck

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FOREWORD

The first edition of this masterfully written and edited compendium on the subject of primary immunodeficiency diseases contained descriptions of about 70 entities. The second edition, published 8 years later, described about 120 distinct primary immunodeficiency diseases. The current third edition, in 2013, details about 250 conditions. This exponential increase in the recognition of errors in host defense has occurred in diminishing intervals of time, attesting to the rapid advances in genetics, molecular biology, and cell biology and the equally fast application of basic science in clinical medicine. In examining the recent literature, we have found descriptions of five new immunodeficiencies in one week, so the field continues to grow. Similarly, these advances have begun to move into translational medicine with more frequent, but still early, reports of successful therapies.

It is quite likely that host defense is influenced by hundreds of genes, most of which will be shown to produce disease if altered by mutations, copy number variations, deletions and insertions, or epigenetic phenomena, the latter often caused by environmental influences but still transmissible to future generations. Another pathogenic mechanism is mediated by molecular changes in small RNA species, recently shown to enhance or reduce gene expression. New genetic methods already being applied to the search for causes of Mendelian and complex genetic diseases will permit discovery of new genes influencing the various mechanisms of defense against pathogens. These methods include genome-wide association studies, followed by second- and third-generation sequencing. Specific mutations will be found in patients with frequent infections or autoimmune symptoms by whole-exome and whole-genome sequencing. Other genetic phenomena, such as uniparental disomy and reversion of mutations (first proven in adenosine deaminase deficiency), will also clarify puzzling clinical findings in patients.

A field of importance to further understanding of the development of our normal immune response and its failures is the human microbiome, especially that found in the intestine. It is now clear that the bacterial, viral, and fungal populations in our bodies are several logs higher than the number of cells making up our bodies. The intestinal microbiome is made up of numerous species, many previously unknown and not amenable to culture, but now discoverable by novel genetic methods. These unicellular organisms apparently are critical in the development of our normal host defense, and alterations of their diverse makeup are instrumental in the pathology of autoimmunity and at least some examples of defects in host defense.

All in all, it is becoming clear that immunology and host defenses in general, as is true for perhaps all medical specialties and subspecialties, are controlled and altered by genetics, and that immunodeficiency diseases are but results of the power of the infinite variation and fluidity of our genome. It may be that future editions of this important subject perhaps should be expanded to "Abnormalities of Host Defense," of which immunodeficiency is an important component.

A potentially even more important outcome of the studies so well described in this book and those to come is a greater understanding of immunology and host defense in general. By studying the interaction of the many genes described with the application of system biology, understanding the role of modifiers, enhancers, and inhibitors will lead to possible strategies of therapeutic intervention. The chapters on susceptibility and resistance to specific infectious diseases provide cogent examples of the beginning of such possibilities.

Finally, as already recognized by Drs. Good and Cooper in the forewords of the first two editions, and greatly expanded in this volume, the roles of innate and adaptive immunity, of phagocytes, and of complement in host defense vastly broaden our understanding of this fascinating field.

Kurt Hirschhorn and Rochelle Hirschhorn
New York, August 2012

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FOREWORD TO THE FIRST EDITION

Modern immunology can be considered to have been launched in 1952, when Colonel Ogden Bruton described an 8-year old boy who, from 2 years of age, experienced recurrent, life-threatening infections including episodes of bacterial pneumonia and sep-ticemia. Using the newly-introduced technique, serum elec-trophoresis, Bruton found the boy to be agammaglobulinemic. When challenged with antigens, he failed to produce specific antibodies. Upon treatment by passive immunization with large doses of intramuscularly-injected gammaglobulin, his susceptibility to infections was dramatically terminated. Detailed investigations of similar patients, by Charles Janeway in Boston and my group in Minneapolis, demonstrated many similarly affected children, and proved that agammaglobulinemia was often an X-linked, inherited disorder.

In the course of caring for agammaglobulinemic patients, we realized that they were especially susceptible to encapsulated bacterial pathogens, including *Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Pseudomonas aeruginosa,* and to a lesser extent, *Staphylococcus aureus*. In contrast however, they could impressively resist infections caused by fungi, coliforms, tuberculosis, bacillus Calmette-Guérin (BCG), and many viruses such as measles, chicken pox, rubella, and vaccinia. Thus, the susceptibility profile of agammaglobulinemic patients bisected the microbial universe. As an experiment of nature, patients with X-linked agammaglobulinemia (XLA) introduced us to additional, crucially important concepts concerning how plasma cells and lymph node germinal centers, which are lacking in agammaglobulinemic patients, must be the source of antibodies providing resistance to encapsulated bacterial pathogens. We reasoned that distinct mechanisms of defense that were intact in agammaglobulinemic patients must have been designed to protect against other types of infections. Lymphocytes in the deep paracortical regions of lymph nodes, which appeared normal in XLA patients, were found to mediate this second type of immune protection, cellular immunity, which was later shown to be dependent on the thymus. This conclusion was partially derived from the study of a different group of patients, those with DiGeorge syndrome, who had congenital absence of the thymus.

Thus, it was evident from the beginning that patients with immunodeficiencies, as experiments of nature, helped us to bisect not only the microbial universe, but also the universe of lym-phoid cells and the universe of immunological responses. Further investigations throughout the 1960s, 1970s, and 1980s confirmed that this compartmentalization related to the fundamental lymphocyte dichotomy of B cells versus thymus-dependent T cells. Moreover, patients with different immunodeficiency syndromes helped define the nature and the role in immune responses of other components of the host defense system, such as phagocytes and complement, and to recognize the diseases that occur when these components are absent or not functional.

Over the last decade, advances in molecular biology have allowed for an even greater understanding of the immune system, and the multitude of molecular pathways that regulate growth, differentiation, communication, and effector functions within and between cells. In 1993, two groups of researchers, led by David Vetrie and Satoshi Tsukada, discovered that the difficulties of Bruton's patient and other patients with XLA were due to many different mutations of an X-linked gene that encodes a B-cell specific tyrosine kinase, Btk. In the few years since that discovery, the molecular genetic universe has expanded phenomenally, so that almost every month there is news of the identification of another immune disease gene.

The present volume, edited by Professors H. D. Ochs, C.I.E. Smith, and J. M. Puck, is the first comprehensive guide to this new molecular genetic universe. Herein, diseases of the immune system are presented and analyzed, both in terms of their clinical features and in the context of the impressive molecular and genetic definitions which can be put forward in 1998. Over 90 well-defined primary diseases of the human immune system are listed in the introductory chapter of this book; specific diseases are discussed in later chapters organized by syndrome (Part II). The current understanding of each disorder is outlined, including discussions of clinical issues and clinical presentation, infections, genetic mutations, protein function, cell biology, and management. Framing these discussions of individual diseases are two equally modern presentations—first, a section of seven chapters outlining the essential concepts of immunology and genetics needed to understand primary immunodeficiency diseases, and at the end, a section covering the most current approaches to assessment and treatment of patients with these conditions. Each authoritative chapter is written by a world leader in the field, or in many cases by a pair or group of immunological specialists with complementary perspectives, to present the most up-to-date and complete information available.

This book is an impressive demonstration of how far we have come. Recent studies of primary immunodeficiency diseases have, perhaps more than any other group of diseases, revealed the power of modern molecular genetics to define diseases in precise molecular terms. This approach has already suggested therapeutic possibilities which have proven successful; it has also set the stage for testing gene therapies meant to cure primary immunodeficiency diseases at the molecular level. Just how disruptions of Btk account for all of the morphological and immunological abnormalities and disease susceptibilities of patients with XLA has not yet been elucidated, but future work will show how this molecule interacts with other gene products in the B lymphocyte. Studying XLA will continue to reveal fundamental issues in lymphology and immunobiology.

The knowledge of primary immunodeficiency diseases reflected in this volume continues to grow, based on insights derived from the study of individuals with primary immunodeficiency exemplified by Bruton's original agammaglobulinemic patient. Analysis of each of the immune system diseases in its own way represents the molecular interpretation of an informative experiment of nature. In the aggregate, these analyses help us understand more deeply how man can exist free of infection while living in a veritable sea of microorganisms. This volume constitutes a milestone, marking where we now stand and indicating where we are heading, as we continue to interpret lessons in a most constructive fashion from the greatest teachers of modern immunology: patients with primary immunodeficiency diseases.

Robert A. Good, M.D., Ph.D., D.Sc.
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July 1998

FOREWORD TO THE SECOND EDITION

The primary immunodeficiency diseases, the first of which were recognized over 50 years ago, are now generally appreciated as major health problems by affected patients, their families, physicians, and even the general public. In 1999, this book was the first comprehensive compendium devoted to primary immunodeficiency diseases. While most are relatively rare, some of these conditions, like IgA deficiency and common variable immunodeficiency, occur with a frequency that makes these patients likely to be seen by most physicians.

The study of patients with these genetically determined immune disorders in conjunction with the study of animal models has led to remarkable progress in our understanding of the interacting components of the complex immune system and how they function in humans. As a consequence, earlier recognition and better treatment options are provided for patients with primary immunodeficiency diseases, as well as for the even larger number of individuals with secondary immune deficiency conditions. This authoritative book, now in its second edition, contains a comprehensive account of currently available information. In the short years since the first publication, the number of known immunodeficiency genes has grown from less than 70 to well over 120, reflecting the tremendous expansion of knowledge in this field. The rich base of information contained in these pages makes it clear that there are few fields in medicine in which laboratory-based research and the study of diseases in patients have been so mutually complementary as for the primary immunodeficiency diseases.

The first immunodeficiency diseases to be identified, namely X-linked agammaglobulinemia, and the more clinically severe congenital lymphopenic syndromes were diseases that are now known to reflect compromised development in the effector limbs of the adaptive immune system. Experimental delineation of the developmentally distinct lineages of lymphocytes, the thymus-dependent population of T cells, and the bone marrow-derived B cells, made possible the recognition of their respective roles in cell-mediated and humoral immunity. Accordingly, the primary immunodeficiency diseases were found to belong to distinct classes, those primarily affecting T cell development, like the thymic underdevelopment seen in the DiGeorge syndrome, and those featuring impaired B cell development and antibody production, as seen in Bruton X-linked agammaglobulinemia. Severe combined immunodeficiency (SCID), recognized first by Glanzmann and Riniker, featured instead a developmental failure of both T and B cells. With the ensuing molecular biology revolution, the pace of the genetic analysis of the immunodeficiency diseases quickened remarkably. As more and more details have been learned about the life history of T and B lineage cells, many of the genetically determined defects in these differentiation pathways can now be identified quite precisely in genetic and molecular terms.

As is indicated in the contents of this book, we currently have sufficient information about the lymphocyte differentiation pathways to categorize primary immunodeficiency diseases into gene mutations that affect (1) DNA transcription factors; (2) rearrangement and expression of the T cell receptor (TCR) and immun-oglobulin genes; (3) signal transducing components of the TCR and B cell receptor (BCR) complexes; (4) essential signaling pathway elements employed by TCR and BCR; (5) coreceptor molecules that are essential for normal function of T and B cells; (6) cytokines and cytokine receptors that promote T and B cell production, proliferation, and differentiation; and (7) cell surface molecules that are necessary for normal lymphocyte homing and intercellular interactions in the peripheral lymphoid tissues, including the spleen, lymph nodes, intestinal Peyer's patches, and appendix. It has also become increasingly evident that the normal function of the effector T and B cell populations depends on other types of cells as well. An especially important cell partner is the dendritic cell, because it responds to potential pathogens by presenting antigen to initiate the T cell response and, in turn, the B cell response. Although few primary immunodeficiency diseases have as yet been attributed to developmental flaws in this cell type, impaired dendritic cell function is an important component of the immunodeficiency caused by gene mutations that prevent CD40 expression or expression of the CD40 ligand on T cells.

The specific adaptive immune responses mediated by T and B cells and their collaborators, although essential, are only a part of the overall host defense strategy. There is an ever-growing awareness that innate immunity is equally important and complex. Disorders of the complement system, abnormal function of phago-cytic cells, and deficiencies of the chemokines and chemokine receptors that influence lymphocyte-phagocytic cell interactions can all result in an impaired ability to eliminate pathogens. Natural killer cells with their diverse array of activating and inhibitory receptors are also beginning to be recognized as one of the dysfunctional cell types in some immunodeficiency disorders.

Infections are the major complications of the immunodeficiency diseases, and, as recognized by the late Robert Good, a true giant in the establishment of the field and author of the original forword to the first edition of this book, the types of infections differ according to the specific gaps in host defense. Primary antibody deficiency states predispose to serious bacterial infections, as do certain complement component and neutrophil deficiencies. Viral and fungal infections are particularly notable in patients with T cell dysfunction. Different infectious disease patterns are seen with other host defense defects. For example, mycobacterial and salmonella infections are common in patients who have mutations in the genes for IL-12 or the receptors for IL-12 and interferon-γ, because these signaling molecules are especially important for normal macrophage activation to kill intracellular pathogens. Characterization of the different patterns of infections has been significantly enhanced by the development of databanks devoted to patients with the relatively rare primary immunodeficiency diseases.

Treatment has advanced in parallel with improved diagnosis of immunodeficiency diseases, understanding of their cellular and molecular basis, and better definition of their clinical consequences. Prophylactic antibiotics can be helpful in reducing the frequency of certain types of infections. Immunoglobulin replacement, employed first by Bruton to treat a boy with congenital agammaglobulinemia, has been refined through the development of safe and efficient preparations of intravenous immunoglobulin. Better ways to perform bone marrow transplantation have made this life-saving mode of cellular engineering safer and available to more patients with severe combined immunodeficiency disease. Enzyme replacement can benefit SCID patients with adenosine deaminase deficiency. Finally, gene therapy has proven effective for the cure of two types of SCID, albeit presently with an attendant risk of lymphoproliferative disease. For all too many patients with primary immunodeficiency diseases, however, a cure is still not yet possible and will come only with improved knowledge that must be gained through continued study. In the meantime, early diagnosis remains the key for a quality life for many patients with an immunodeficiency disease. Toward this end, this newly updated book provides a remarkably comprehensive and clinically useful source of information about this challenging group of disorders.

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1.

GENETICALLY DETERMINED IMMUNODEFICIENCY DISEASES: A PERSPECTIVE

C. I. Edvard Smith, Hans D. Ochs, and Jennifer M. Puck

We are in an era of explosive growth in our understanding of the molecular and genetic basis of immune defects. In the early 1990s, only a handful of genes had been associated with primary immunodeficiency disorders (PIDs). By 2000, when the first edition of this book was published, some 60 genes causing PID had been identified. The most recent summary of PID genes (Table 1.1) and their products (Fig. 1.1), with over three times this number, is already incomplete due to the rapid pace of discovery of additional genes that are defective in an ever-broadening spectrum of clinical immune disorders. Advances in basic research in immunology, combined with the increasing ease of determining DNA sequence variants, have greatly facilitated the tasks of finding DNA mutations in PID patients and proving their functional significance. The ability to define genetic diseases of the immune system in molecular terms has made possible improved diagnosis, appreciation of the clinical spectrum, genetic counseling and testing, and, most exciting, new therapeutic strategies including gene therapy. Moreover, the discovery of each previously unknown disease gene feeds back into the pool of scientific knowledge, increasing our understanding of molecular immune networks.

In contrast to many other heritable diseases, PIDs are not obvious at birth but become evident only when the affected individual is exposed to microbes and develops severe infections or responds to self-antigens with autoaggression. Although individually rare, these disorders are treatable and therefore important to detect promptly. The spectrum of diseases is very broad and covers both increased susceptibility to infections and impaired regulation of immune function leading to autoimmunity. The inheritance of PIDs can be recessive or dominant; some produce symptoms early in life, while others are manifested later; and acquired forms of PID associated with somatic mutations are increasingly recognized.

The "classical" PIDs are single gene disorders with either autosomal recessive or X-linked recessive inheritance, such as severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS), or X-linked agammaglobulinemia (XLA). In contrast, dominant inheritance is typically seen when a mutation affects a protein that normally functions in a multimeric complex. For example, in autoimmune lymphoproliferative syndrome (ALPS) caused by heterozygous mutations in the genes encoding Fas or Fas-ligand, both the receptor and its ligand are assembled into homotrimers. For a trimer to be functional, all monomeric components need to be unmutated; dominant interference with the function of protein produced by a normal allele results from having one or two mutated monomers in the assembled complex. If normal and mutated protein monomers are produced in equal amounts, only one out of eight trimeric signaling units will be free from any mutated chains and able to function properly (Chapter 29). Because only a single mutation event is needed and a survival advantage is conferred on cells with defective Fas-mediated apoptosis, de novo somatic mutations of a single *FAS* gene allele have been found capable of causing acquired dominant ALPS (Holzelova et al., 2004). Another example is the hyper-IgE syndrome, where missense mutations impair STAT3 dimer formation, reducing the intracellular JAK-STAT signaling by 75 percent (Chapter 37). Dominant inheritance is also seen in some forms of severe congenital neutropenia, in which a mutated, misfolded protein activates the "unfolded protein response," a series of cellular stress responses that are activated by the accumulation of malformed proteins and can ultimately trigger cellular removal by apoptosis (Chapter 50).

As we have pointed out in previous editions of this book, susceptibility versus resistance to infections can depend on inheritance of variant forms of immune system components. HIV typically uses the CCR5 co-receptor to enter human

Table 1.1 PRIMARY IMMUNODEFICIENCY DISEASES

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
	A. Combined B- and T-Cell Immunodeficiencies			
1. Severe combined immunodeficiency (SCID) without T or B cells (T^-B^-)				
a. SCID with leukocyte deficiency. Reticular dysgenesis. *AK2*	**Stem cell defect affecting maturation of leukocytes, including all lymphocytes; associated deafness**	**AR**	**1p34**	**18**
b. SCID with radiosensitivity. Artemis deficiency. *DCLRE1C*	**DNA cross-link repair 1C protein, Artemis; impaired VDJ recombination of B- and T-cell receptor genes**	**AR**	**10p13**	**13**
c. SCID with RAG1 deficiency *RAG1*	**Recombinase-activating protein 1. Impaired VDJ recombination of B- and T-cell receptor genes (*RAG1* and *RAG2* are adjacent genes).**	**AR**	**11p13**	**13**
d. SCID with RAG2 deficiency *RAG2*	**Recombinase-activating protein 2. Impaired VDJ recombination of B and T cell receptor genes (*RAG1* and *RAG2* are adjacent genes).**	**AR**	**11p13**	**13**
2. SCID with nonfunctional T and B cells hypomorphic mutations in genes associated with SCID				
a. Omenn syndrome with				
i. RAG1 deficiency *RAG1*	**RAG1 partially deficient rearrangement of B- and T-cell receptor genes**	**AR**	**11p13**	**13**
ii. RAG2 deficiency *RAG2*	**RAG2 partially deficient rearrangement of B- and T-cell receptor genes**	**AR**	**11p13**	**13**
iii. Artemis deficiency *DCLRE1C*	**Artemis, partially deficient in VDJ rearrangement**	**AR**	**10p13**	**13**
iv. IL-7Rα deficiency *IL-7Rα*	**IL-7 receptor α chain, partially deficient**	**AR**	**5p13**	**11**
v. ADA deficiency *ADA*	**Adenosine deanimase hypomorphic mutation**	**AR**	**20q13.11**	**14**
vi. DNA ligase IV (LIG4) deficiency *LIG4*	**Ligase IV, hypomorphic mutation**	**AR**	**13q33–34**	**13**
vii. RNase MRP complex defi ciency *RMRP*	**Cartilage-hair hypoplasia (CHH). See D.5.**	**AR**	**9p13**	**37**
viii. *22q11.2* deletion	**Complete DiGeorge**	**AD**	**22q11.2**	**45**
b. DNA ligase IV deficiency *LIG4*	**Ligase IV, ATP-dependent, involved in VDJ recombination**	**AR**	**13q22–q34**	**13**
c. SCID with microcephaly and radiosensitivity due to deficiency of non-homologous end-joining factor 1. *NHEJ1*	**DNA repair factor (XRCC4-like factor; XLF; Cernunnos) involved in non-homologous end-joining**	**AR**	**2q35**	**13**
d. SCID due to deficiency of DNA-PKcs *PRKDC*	**DNA activated protein kinase catalytic subunit (defective in SCID mouse)**	**AR**	**8q11.2**	**13**
3. SCID without T cells (T^-B^+)				
a. X-linked SCID (γc-chain deficiency) *IL2RG*	**Common γ (γc) chain protein, a component of receptors for multiple cytokines (IL-2, -4, -7, -9, -15, and -21)**	**XL**	**Xq13.1**	**10**
b. SCID with JAK3 deficiency *JAK3*	**Janus-activating kinase 3 (JAK3), a cytoplasmic tyrosine kinase interacting with γc to transmit signals from extracellular binding of multiple cytokines**	**AR**	**19p13.1**	**10**
c. SCID with IL-7Rα deficiency *IL7R*	**IL-7 receptor α chain**	**AR**	**5p13**	**11**
d. SCID with CD45 deficiency *PTPRC*	**Protein tyrosine phosphatase receptor type C (PTPRC)**	**AR**	**1q31.3–q32.1**	**12**

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
e. SCID with CD3DEZ chain deficiency *CD3 δ/ε/ξ*	CD3δ, ε, or ξ component of CD3 antigen receptor complex. Required for thymic T-cell maturation.	AR	11q23	11
f. Human Nude/SCID *FOXN1*	Forkhead box N1 protein, winged-helix-nude (*Whn*). Transcription factor required for thymus and hair follicle development.	AR	17q11.2	21
g. SCID due to absent coronin-1A *CORO1A*	Required for lymphocyte migration and exit from the thymus	AR	16p11.2	23
4. Deficiencies of purine metabolism				
a. SCID with ADA deficiency *ADA*	Adenosine deaminase (ADA) is required for purine metabolism; elevated purine metabolites are toxic to T and also B and NK cells.	AR	20q13.11	14
b. SCID with PNP deficiency *PNP*	Purine nucleoside phosphorylase (PNP) is required for purine metabolism; elevated purine metabolites are toxic to T and B cells.	AR	14q11.2	14
5. Calcium channel deficiency (normal number of T cells with defective TCR-mediated activation)				
a. ORAI-1 deficiency *ORAI1*	Defect in Ca^{++} release-activated channel (CRAC) modulatory component; autoimmunity, myopathy	AR	12q24.31	20
b. STIM1 deficiency *STIM1*	Defect in stromal interaction molecule Ca^{++} sensor (STIM); autoimmunity, myopathy	AR	12p13.33	20
6. MHC class II (major histocompatiblity complex: class II) deficiency secondary to deficiencies of transcription factors for MHCII expression				
a. CIITA deficiency *MHC2TA*	MHCII transactivator (CIITA) protein, a non-DNA binding component of the MHCII promoter-binding complex; complementation group *A*	AR	16p13	16
b. RFXANK deficiency *RFXANK*	Regulatory factor X-associated ankyrin-containing protein (RFXANK), an MHCII promoter-binding protein; complementation group B	AR	19p12	16
c. RFX-5 deficiency *RFX5*	MHCII promoter X box regulatory factor 5 (RFX5), an MHCII promoter-binding protein; complementation group C	AR	1q21	16
d. RFXAP deficiency *RFXAP*	Regulatory factor X-associated protein (RFXAP), an MHCII promoter-binding protein; complementation group D	AR	13q	16
7. MHC class I deficiency				
a. TAP1 deficiency *TAP1*	Transporter protein associated with antigen presentation 1 (TAP1)	AR	6q21.3	17
b. TAP2 deficiency *TAP2*	Transporter protein associated with antigen presentation 2 (TAP2)	AR	6q21.3	17
c. Tapasin deficiency *TAPBP*	TAP binding protein (tapasin)	AR	6p21.3	17
8. Class-switch recombination defect (hyper-IgM syndromes) affecting both B and T cells; see also B.6.				
a. CD40L deficiency *TNFSF5*	CD40 ligand (CD40L, CD154). Tumor necrosis factor superfamily member 5 (TNFSI5).	XL	Xq26	26
b.CD40 deficiency *TNFRSF5*	CD40. Tumor necrosis factor receptor superfamily member 5 (TNFRS5).	AR	20q13.12	26

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
9. Non-SCID CD3 deficiency due to absence of proteins forming the CD3 complex required for T-cell receptor signaling See A.2.e for SCID with CD3δέζ deficiency.				
a. CD3ε deficiency *CD3E*	CD3ε polypeptide	AR	11q23	11
b. CD3γ deficiency *CD3G*	CD3γ polypeptide	AR	11q23	11
c. CD3ζ deficiency *CD3Z*	CD3ζ polypeptide (CD247)	AR	1q242.2	11
10. CD8 deficiency *CD8A*	CD8 antigen, α polypeptide (p32)	AR	2p12	19
11. ZAP-70 deficiency *ZAP-70*	Cytoplasmic tyrosine kinase ZAP-70 (T-cell receptor ζ-chain associated protein kinase, 70kDa). Signaling from the T-cell receptor during T-lineage development.	AR	2q12	15
12. IL-2 R α deficiency *IL2RA*	IL-2 receptor α-chain (IL-2Ra, CD25) is required for regulation and control of autoreactive T cells. Mutations cause an IPEX-like phenotype (see D.3b) with SCID features.	AR	10p15.1	10
13. p56 Lck deficiency *LCK*	Lymphocyte-specific protein tyrosine kinase. Required for T-cell maturation in the thymus.	AR	1p34.3	32; Hauck F et al. JACI 2012;130:1144.
14. IKAROS deficiency *IKAROS*	IKAROS is a hematopoietic specific zinc-finger transcription factor and a regulator of lymphoid development. The only case reported had anemia, neutropenia, and thrombocytopenia and lacked B cells.	AD de novo	7p12.2	Goldman FD et al. Pediatr Blood Cancer 2012;58:591.
15. Cartilage hypoplasia *RMRP*	Abnormal T-cell number and function. See D.5.	AR	9p13	37
RNase MRP complex deficiency *RMRP*	Cartilage-hair hypoplasia (CHH). See D.5.	AR	9p13	36
16. STAT5b deficiency *STAT5b*	Growth hormone insensitive. Dwarfism, lymphopenia, interstitial pneumonia (see F.5), IPEX-like phenotype. See also D.3.b.	AR	17q21	32
17. ITK deficiency *ITK*	Lymphopenia, recurrent EBV infections. See also E.8.i.	AR	5q33.3	Huck K et al. J Clin Invest 2009;119:1350.
18. CD27 deficiency *CD27 (TNFRSF7)*	CD27 is a co-stimulatory molecule that regulates lymphocyte differentiation. Combined immune deficiency and hypogammaglobulinemia (see also B.4.i).	AR	12p13.3	Van Montfrans JM et al. JACI 2012; 129:787.
19. Serine threonine kinase 4 (STK4) deficiency (also known as MST1) *STK4*	Lymphopenia and neutropenia due to enhanced apoptosis of naïve and proliferating T lymphocytes resulting in recurrent bacterial and viral infections, including persistent EBV viremia and EBV+ B-cell lymphoma. See also C.2.	AR	20q13.12	Abdollahpour H et al. Blood 2012;119:3450, 2012; Nehme NT et al. Blood 2012;119:3458.
B. Deficiencies Predominantly Affecting Antibody Production				
1. Agammaglobulinemia				
a. XLA (X-linked agammaglobulinemia) *BTK*	Bruton agammaglobulinemia tyrosine kinase (BTK) required for intracellular signaling in B-cell development	XL	Xq21.3	25
b. X-linked hypogammaglobulinemia with growth hormone deficiency	BTK not affected; gene defect unknown	XL	—	25
c. μ heavy-chain deficiency *IGHM*	μ heavy chain. Part of the pre-B and B-cell receptor complex. Required for development of B cells from B-lineage progenitors.	AR	14q32.3	25
d. λ5 surrogate light-chain deficiency *IGLL1*	λ5 surrogate light chain. Part of receptor complex on pre-B cells required for B-lineage differentiation.	AR	22q11.22	25

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
e. Igα deficiency *CD79A*	Ig-associated α chain signaling component of pre-B and B-cell receptor complex required for B-lineage differentiation and B-cell signaling	AR	19q13.2	25
f. Igβ deficiency *CD79B*	Ig-associated β chain signaling component of pre-B and B-cell receptor complex required for B-lineage differentiation and B-cell signaling	AR	17q23	25
g. BLNK deficiency *BLNK*	B-cell linker/SLP-65/BASH. B-cell signaling protein.	AR	10q24.1	25
h. PI3K deficiency *PIK3R1*	A mutation in *PIK3R1* resulted in absence of p85a, a regulatory subunit of PI3K; absence of pro-B and B cells and reduced numbers of NK cells.		5q13.1	Conley ME et al. J Exp Med 2012;209:463.
i. LRRC8 deficiency *LRRC8*	Leucine-rich repeat containing 8 (LRRC8) transmembrane protein required for B-cell development. Single case.	AD	9q34.2	Sawada et al. J Clin Invest 2003;112:1707.
j. Thymoma with hypogammaglobulinemia	Lack of B lymphocytes and immunoglobulins	Acquired, usually sporadic	—	Good et al. Surgery 1956;40:1010.
2. Selective deficiency of Ig isotypes/subclasses due to isolated or combined deficiencies				
a. IgA deficiency	Failure of IgA B-cell differentiation	Complex	—	28
b. α1 subclass deficiency *IGHA1*	IgA1 is the major IgA subclass.	AR	14q32.33	28
c. α2 subclass deficiency *IGHA2*	IgA2 is mainly found in the gastrointestinal tract.	AR	14q32.33	28
d. γ1 subclass deficiency *IGHG1*	IgG1 constitutes 65 percent of serum IgG.	AR	14q32.33	28
e. γ2 subclass deficiency *IGHG2*	IgG2 constitutes 25 percent of serum IgG.	AR	14q32.33	28
f. γ3 subclass deficiency *IGHG3*	IgG3 constitutes 8 percent of serum IgG. Partial IgG3 deficiency is associated with the "g" allotype and caused by reduced isotype switching.	AR	14q32.33	28
g. γ4 subclass deficiency *IGHG4*	IgG4 constitutes 4 percent of serum IgG.	AR	14q32.33	28
h. IgG subclass deficiency with IgA deficiency	Defect in differentiation of a B lymphocyte subset or in expression of IgG	Unknown	—	28
i. ε isotype deficiency *IGHE*	IgE is encoded by a single gene.	AR	14q32.33	28
3. Light-chain deficiency				
a. κ light-chain deficiency *IGKC*	κ light chain binds to a heavy chain to form immunoglobulins.	AR	2p11	Stavnezer-Nordgren et al. Science 1985;230:458.
4. Common variable immunodeficiency and related disorders (for WHIM see F.8)				
a. Common variable immunodeficiency of unknown origin	Serum IgG low, IgA low or absent, IgM variable. Variable impairment of T-cell function.	Complex	—	28
b. ICOS (inducible T-cell costimulator) deficiency *ICOS*	ICOS is expressed by activated T cells and interacts with ICOSL (B7RP-1). Deficiency results in late-onset B-cell loss.	AR	2q33	28
c. CD19 deficiency *CD19*	CD19 molecule expressed by B cells	AR	16p11.2	28
d. CD20 deficiency *MS4A1*	Membrane-spanning 4 domains, subfamily A, member 1	AR	11q12.2	28

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
e. CD81 deficiency *CD81*	CD81 molecule expressed by B cells	AR	11p15.5	28
f. TACI deficiency *TNFRSF13B*	Tumor necrosis factor receptor superfamily member 13B	AD, AR	17p11.2	28
g. BAFF receptor deficiency *TNFRSF13C*	Tumor necrosis factor receptor superfamily member 13C	AR	22q13.2	28
h. CD21 deficiency *CD21/CR2*	CD21/complement component receptor 2 (CR2) is part of the B-cell co-receptor and the EBV receptor.	AR	1q32.2	28
i. CD27 deficiency *CD27 (TNFRSF7)*	CD27, a type I transmembrane protein, belongs to the TNFR family and interacts with its ligand, CD70. CD27 regulates differentiation, survival, and function of lymphocytes. Defective T-cell-dependent antibody responses associated with hypogammaglobulinemia and persistent symptomatic EBV infection.	AR	12p13.3	Van Monfrans JM et al. JACI 2012;129:787.
j. LRBA deficiency *LRBA*	Lipopolysaccharide responsive beige-like anchor protein (LRBA). Early-onset hypogammaglobulinemia due to defective B-cell development/activation and autophagy, and increased apoptosis.	AR	4q13	Lopez-Herrera G et al. AJHG 2012;90:986.
5. Other antibody deficiencies				
a. Antibody deficiency with normal immunoglobulin levels	Defective antigen-specific antibody production	Unknown	—	56
b. Transient hypogammaglobulinemia of infancy	Delayed maturation of T-cell helper function	Unknown	—	Gitlin and Janeway. Prog Hematol 1956;1:318.
6. Defects of class-switch recombination and somatic hypermutation (hyper-IgM syndromes) affecting B cells; see also A.8.				
a. AID deficiency *AICDA*	Activation-induced cytidine deaminase	AR	12p13	27
b. UNG deficiency *UNG*	Uracil-DNA glycosylase	AR	12q24.11	27
c. Selective deficiency in Ig class-switch recombination	Defect downstream of AID, normal somatic hypermutation	Unknown	—	27
d. PMS2 deficiency *PMS2*	DNA repair defect. See G.4	Unknown	—	27
C. Defects in Lymphocyte Apoptosis				
1. Autoimmune lymphoproliferative syndrome (ALPS), characterized by lymphadenopathy, splenomegaly, cytopenias, increased risk of B-cell lymphoma; see also D.3.				
a. ALPS-FAS (defective CD95) *FAS* or *TNFRSF6*	Apoptosis mediator CD95 (Fas/APO-1) required for lymphocyte homeostasis induces apoptosis via engagement of FasL.	AD, AR	10q23.31	30
b. ALPS-FASL (defective CD178) *FASL* or *TNFSF6*	Fas ligand (FasL) induces apoptosis via engagement of Fas.	AD	1q24.3	30
c. ALPS-CASP10 Caspase 10 deficiency *CASP10*	Apoptosis-related cysteine protease. *CASP8* and *CASP10* are adjacent genes.	AR	2q33.1	30
d. CEDS (caspase 8 deficiency state) *CASP8*	Caspase 8; apoptosis-related cysteine protease; in addition to ALPS phenotype, these patients have defective T-, B-, and NK-cell activation and recurrent infections.	AR	2q33.1	30
e. RALD (Ras-associated autoimmune leukoproliferative disorder) *NRAS*	Neuroblastoma Ras viral oncogene homolog (NRAS) sporadic somatic mutations in NRAS	AR	1p13.2	1, 30

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
f. V-KI-RAS2 Kirsten rat sarcoma viral oncogene homolog (KRAS) *KRAS*	Sporadic somatic mutations in KRAS, similar to NRAS	AR	12p12.1	1, 30
2. Increased apoptosis affecting naïve T cells due to MST1 deficiency *STK4*	Serine-threonine protein kinase-4 (STK4) encodes mammalian sterile 20-like 1 (MST1), which phosphorylates FOXO transcription factors. MST1-deficient naïve T cells express decreased amount of FOXO1, IL-7R, and BCL2 and increased FAS expression and are progressively reduced in number. Recurrent bacterial and viral infections, autoimmunity. See also A.19.	AR	20q13.12	Abdollahpour H. et al. Blood 2012;119:3450; Nehme NT et al. Blood 2012;119:3458.
D. Other Well-Defined Immunodeficiency Syndromes				
1. Wiskott-Aldrich syndrome (WAS), X-linked thrombocytopenia (XLT), and X-linked neutropenia (XLN) *WAS*	WAS protein (WASp), expressed only in hematopoietic cells. Classic WAS is characterized by thrombocytopenia, small platelets, eczema, autoimmune diseases, lymphoma; XLT is a mild form of WAS; XLN (only neutropenia) is caused by missense mutations in the GTPase binding domain of WASp.	XL	Xp11.22	43
2. WASp-interacting protein (WIP) deficiency *WIPF1*	WIP is complexed with WASp and stabilizes WASp. If WIP is not expressed, WASp is absent, resulting in a phenotype similar to classic WAS (only a single case reported).	AR	2q31.1	43
3. Autoimmune disorders; see also C.1.				
a. Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED) *AIRE*	Autoimmune regulator-1 (AIRE-1) protein is a transcription factor expressed in the thymus. Autoantibodies to IL-17A, IL17-F, IL-22 are often present.	AR	21q22.3	31
b. Immune deficiency/dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) *FOXP3*	Forkhead box P3 transcription factor (FOXP3). Expressed by $CD4^+$ $CD25^+$ regulatory T cells (Treg); multiple autoimmune findings include cytopenias, type 1 diabetes, thyroiditis, villous atrophy, eczema.	XL	Xp11.23	32
4. DiGeorge/velo-cardio-facial syndrome (22q11.2 deletion syndrome)	Multiple congenital anomalies most often due to large deletion (3 Mb) in 22q11.2 (or less frequently a deletion in 10p). Defect in thymic development. May be associated with conotruncal malformation, hypoparathyroidism, and other congenital anomalies.	AD or de novo defect	22q11.2	45
5. CHH (cartilage-hair hypoplasia) *RMRP*	RNA component of mitochondrial RNA-processing endoribonuclease (RMRP). Short-limbed dwarfism, metaphyseal dysostosis, sparse hair, bone marrow failure, autoimmunity, lymphoma or other cancers.	AR	9p13	37
6. Immuno-osseous dysplasia—Schimke syndrome *SMARCAL1*	Short stature, spondyloepiphyseal dysplasia, nephropathy; bacterial, viral, fungal infections, bone marrow failure. May present as SCID.	AR	2q35	Boerkoel CF et al. Eur J Pediatr. 2000;159:1.
7. Hyper-IgE syndromes (HIES)				
a. AD-HIES (Job syndrome) due to STAT3 deficiency *STAT3*	Dominant negative heterozygous mutations in STAT3. Distinct facial features, eczema, osteoporosis and fractures, scoliosis, delayed shedding of primary teeth, hyperextensible joints, abscesses, due to *Staphylococcus aureus*, pneumatoceles, candida infections, elevated IgE, decreased Th17 cell numbers, decreased memory B cells.	AD, or de novo defect	17q21.2	38

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
b. AR-HIES due to *DOCK8*-deficiency *DOCK8*	Detection of cytokinesis 8 (Dock8). Recurrent respiratory infections, viral and staphylococcal skin infections, increased risk of cancer. Combined T- and B-cell defects.	AR	9q24.3	38
c. AR-HIES due to TYK2 deficiency *TYK2*	Tyrosine kinase 2 (TYK2). Susceptibility to intracellular bacteria, fungi, viruses.	AR	19p13.2	38
8. Chronic mucocutaneous candidiasis (CMC)				
a. CARD9 deficiency *CARD9*	Chronic mucocutaneous candidiasis (CMC) involving skin and mucous membranes, invasive candidasis and dermatophytosis due to mutations in caspase recruitment domain-containing protein 9 (CARD9).	AR	9q34.4	22
b. IL-17RA deficiency *IL-17RA*	IL-17 signaling pathway in monocytes, epithelial cells, and fibroblast. Cellular responses to IL-17A/F are abolished. CMC.	AR	22q11.1	Puel A et al. Science 2011;332:65.
c. IL-17F deficiency *IL-17F*	IL-17F is expressed as dimers on T cells. CMC.	AD	6p12	Puel A et al. Science 2011;332:65.
d. STAT1 deficiency *STAT1*	Gain-of-function heterozygous mutations of STAT1 interfere with nuclear dephosphorylation of activated STAT1. Impaired development of IL-17-producing T cells. CMC. IPEX-like phenotype (see D.3.b; for loss of function, see F.4).	AD	2q32.2	Liu L et al. J Exp Med 2011;208:1635.
9. Comel-Netherton syndrome *SPINK5* (serine protease inhibitor, Kazal type 5)	Mutations in the serine protease inhibitor LECTI, expressed in epithelial cells. Congenital icthyosis, bamboo hair, allergies, failure to thrive, elevated IgA and IgE, antibody deficiency.	AR	5q32	Renner ED et al. JACI 2009;124:536.
10. Hepatic veno-occlusive disease with immunodeficiency (VODI) *SP110*	Mutations in the nuclear body protein SP110. PCP pneumonia, CMV, Candida, hepatosplenomegaly, thrombocytopenia.	AR	2q37.1	39
11. Dyskeratosis congenita (DKC) 1 (Hoyeraal-Hreidarsson syndrome) *DKC1*	Mutations in dyskerin 1 (DKC1). Nail dystrophy, recurrent infections, ulcerative lesions of mucous membranes, pancytopenia (myelodysplastic), cancer.	XL	Xq28	Jyonouchi S et al. Pediatr Allergy Immunol 2001;22:313.
	E. Defects of Phagocyte Development and Function			
1. Defects of neutrophil differentiation				
a. Severe congenital neutropenia 1 (SCN1) due to ELANE deficiency *ELANE*	Myeloid differentiation	AD	19p13.3	51
b. Severe congenital neutropenia 2 (SCN2) due to GFL1 deficiency *GFL1*	Loss of expression of ELANE (rare), thus interfering with myeloid differentiation	AD	1p22.1	Person RE et al. Nat Genet 2003;34:308.
c. Severe congenital neutropenia 3 (SCN3), Kotsmann *HAX1*	HAX1 regulates apoptosis.	AR	1q21.3	
d. Severe congenital neutropenia 4 (SCN4) due to G6PC3 deficiency *G6PC3*	Glucose-6-phosphatase (G6PC3), myeloid differentiation, enhanced apoptosis of neutrophils		17q21.31	51
e. Glycogen storage disease type 1b *G6PT1*	Glucose-6-phosphate transporter 1 (G6PT1)	AR	11q23.3	51

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
f. Cyclic neutropenia due to ELANE deficiency *ELANE*	Oscillation in all leukocyte and platelet numbers	AD	19p13.3	51
g. X-linked neutropenia due to WASp deficiency *WAS*	Release of myeloid cells from bone marrow. See D.2.	XL	Xp11.22	43
h. LAMTOR2 deficiency *LAMTOR2* (also known as *MAPBPIP*)	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 2. Mutations in P14 cause neutropenia, hypogammaglobulinemia, partial albinism.	AR	1q22	51
i. Shwachman-Bodian Diamond syndrome *SBDS*	Highly conserved protein of unknown function. Pancreatic insufficiency and bone marrow dysfunction, including neutropenia.	AR	7q11.21	Boocock GR et al. Nat Genet 2003;33:97.
j. Warts, hypogammaglobulinemia, recurrent bacterial infections, and "myelokathexis" (WHIM) *CXCR4*	Chemokine C-X-C motif receptor 4 (CXCR4), gain-of-function mutations, hypogammaglobulinemia, reduced B-cell number, severe neutropenia, warts/HPV infections	AD	2q21	40
2. Defects of the intracellular oxidative burst (chronic granulomatous disease [CGD])				
a. X-linked CGD due to cytochrome b beta (CYBB) deficiency *CYBB*	Cytochrome phagocytic oxidase (phox) gp91^{phox}. Together with p22^{phox} forms the heterodimer cytochrome b-245 beta-polypeptide.	XL	Xp11.4	52
b. AR-CGD due to cytochrome b alpha (CYBA) deficiency *CYBA*	Cytochrome oxidase p22^{phox}	AR	16q24	52
c. AR-CGD due to neutrophil cytosol factor 1 (NCF1) deficiency *NCF1*	Cytochrome oxidase p47^{phox}	AR	7q11.23	52
d. AR-CGD due to neutrophil cytosol factor 2 (NCF2) deficiency. *NCF2*	Cytochrome oxidase p67^{phox}	AR	1q25	52
3. Glucose 6-phosphate dehydrogenase deficiency *G6PD*	Granulocyte intracellular killing defect associated with complete absence of G6PD in phagocytes	XL	Xq28	50
4. Myeloperoxidase (MPO) deficiency *MPO*	MPO is required to convert H_2O_2 to hypohalous acid. Intracellular killing of fungi is impaired.	AR	17q23.1	Lehrer RI, Cline MY. J Clin Invest 1969;48:1478; Nauseef WM et al. J Biol Chem 1994;269:1212.
5. Leukocyte adhesion defects (LAD)				
a. LAD1 due to beta-2 integrin deficiency *ITGB2*	Lack of the CD18 cell surface protein leads to defective cell adherence and chemotaxis. Cell surface adhesion complex (CD11a, b, c/CD18) requires integrin β2 (CD18) to be stably expressed. Patients present with leukocytosis, skin ulcers, peridontitis.	AR	21q22.3	53
b. LAD2 due to fucose transporter 1 deficiency *SLC35C1*	Fucose transporter required for proper carbohydrate addition; patients cells lack sialyl-Lewis X and have the Bombay RBC phenotype. Mild LAD1 phenotype with mental retardation, defective neutrophil rolling, and chemotaxis.	AR	11q11.2	53
c. LAD3 *KINDLIN3*	Defect in Rap 1- activation of β 1–3 integrins, mild LAD1 phenotype with bleeding tendency	AR	11q13.1	53

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
d. LAD with RAC2 (regulation of actin polymerization) deficiency *RAC2*	RAS-related, Rho family small GTP-binding protein RAC2. Predominant in neutrophils, involved in $O2^-$ production, actin cytoskeleton. Abnormal adherence and chemotaxis.	AR	22q13.1	53
6. Immunodeficiency with hypopigmentation				
a. Chediak-Higashi syndrome *LYST*	Lysomal trafficking (LYST) regulator. Required for formation of lysosomes and cytoplasmic granules. Partial albinism, recurrent infections, encephalopathy, neutropenia, low NK and CTL activity.	AR	1q42.3	54
b. Griscelli syndrome type 1 *MYO5A*	Myosin-VA (5A) (MY05A) is involved in organelle transport. Partial albinism, neurologic impairment.	AR	15q21.2	54
c. Griscelli syndrome type 2 *RAB27A*	Rab27A is a GTPase that promotes docking of cytotoxic vesicles to the cell membrane. Myosin-VA (5A) and Rab27A are closely linked on chromosome 15q21. Partial albinism, encephalopathy, low NK and CTL activity.	AR	15q21.3	54
d. Hermansky-Pudlak syndrome type 2 *AP3B1*	β subunit of the AP-3 complex (AP3B1). Partial albinism, neutropenia, low NK and CTL activity.	AR	5q14.1	51
7. Glycogen storage disease type Ib *SLC37A4*	Solute carrier family 37 (glycerol-6-phosphate transporter), member 4. Neutropenia, impaired neutrophil migration due to defective glucose 6-phosphate translocase.	AR	11q23.3	Hiraiwa H et al. J Biol Chem 1999;274:5532.
8. Familial hemophagocytic lymphohistiocytosis (FHL) and lymphoproliferative syndromes				
a. FHL1	FHL with unknown gene defect	AR	—	54
b. FHL2 due to perforin deficiency *PRF1*	Perforin 1 (pore-forming protein), a major cytolytic protein	AR	10q22	54
c. FHL3 UNC13D (Munc 13-4) deficiency *UNC13D*	Vesicle priming protein unc-13 homolog D (*C. elegans*). Required to prime vesicles for fusion.	AR	17q25.3	54
d. FHL4 due to syntaxin11 deficiency *STX11*	STX11 is required for fusion of secretory vesicles with the cell membrane.	AR	6q24.2	54
e. FHL5 due to syntaxin11 binding protein 2 (Munc 18-2) deficiency *STXBP2*	STXBP2 has similar functions as STX11.	AR	19p13.2	54
f. X-linked lymphoproliferative syndrome (type 1, XLP1) *SH2D1A*	SH2 domain 1A protein (also called SLAM-associated protein, SAP) is involved in intracellular signaling of T and NK cells. Symptoms, triggered by EBV and other viral infections, include hepatitis, hemophagocytic syndrome, lymphoma, hypogammaglobulinemia, low NKT cells.	XL	Xq25	44
g. X-linked lymphoproliferative syndrome type 2 (XLP2) *XIAP*	Inhibitor of apoptosis, X-linked (XIAP); symptoms are similar to XLP11 and triggred by EBV infections; include splenomegaly, hepatitis, hemophagocytic syndrome, colitis.	XL	Xq25	44
h. Immunodeficiency X-linked, with magnesium defect, EBV infection and neoplasm (XMEN) *MAGT1*	Magnesium transported 1 susceptibility to EBV and other viral infections, respiratory and gastrointestinal infections, lymphoma	XL	Xq21.1	Li FY et al. Nature 2011;475:471.
i. IL2 inducible T-cell kinase (ITK) deficiency *ITK*	EBV-associated lymphoproliferation	AR	5q33.3	Huck K et al.. J Clin Invest 2009;119:1350.

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
j. CD16 deficiency *FCGR3A*	CD16 plays a role in NK cell-mediated cytotoxicity in association with CD2. Persistent herpes virus and human papillomavirus infections; EBV-driven Castleman disease.	AR		Grier JT et al. J Clin Invest 2012;122:3769.
	F. Defects of the Innate Immune System: Receptors and Signaling Components			
1. Interferon-γ receptor deficiency.				
a. IFNγ receptor 1 deficiency *IFNGR1*	IFNγ-receptor 1 (or α-chain) is required for binding IFNγ as well as signaling by associating with JAK1. Susceptibility to mycobacteria and salmonella.	AR, AD	6q23.3	35
b. IFNγ receptor 2 deficiency *IFNGR2*	IFNγ-receptor 2 (or β-chain) is required for signaling by associating with JAK2. Susceptibility to mycobacteria and salmonella.	AR	21q22.11	35
2. IL-12p40 deficiency *ILI2B*	Interleukin-12, 40 KD subunit of IL-12 and IL-23, which is required for the production of IFNγ by T, monocytes, and NK cells. Susceptibility to mycobacteria and salmonella.	AR	5q33.3	35
3. IL-12 and IL-23 receptor β1 chain deficiency *ILI2RB*	Receptor β 1 chain of IL-12 and IL-23, required for IFNγ production. Susceptibility to mycobacteria and salmonella.	AR	19p13.1	35
4. STAT1 deficiency *STAT1*	Signal transducer and activator of transcription 1, 91 KDa. Loss of function mutations involved in IFNγ signaling. Susceptibility to mycobacteria and salmonella. For gain of function, see D.3.b (IPEX-like) and D.8.d (CMC).	AD	2q32.2	35
5. STAT5b deficiency *STAT5B*	Signal transducer and activator of transcription 56, 80 KDa. Immunodeficiency and growth hormone insensitivity. Involved in FOXP3 expression (may present as IPEX-like phenotype).	AR	17q21	32
6. IRAK-4 deficiency *IRAK4*	Interleukin-1 receptor-associated kinase 4, a component of TLR and IL-1R pathway. Recurrent pyrogenic infections.	AR	12ql2	36
7. MYD88 deficiency *MYD88*	Myeloid differentiation primary response gene 88 (MYD88) is a component of the TLR and IL-1R pathway. Recurrent pyrogenic infections.	AR	3p22.2	36
8. X-linked anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) *IKBKG*	NF-κB essential modulator (NEMO), a regulator of NF- κB activation. Anhidrotic ectodermal dysplasia, mycobacterial and pyogenic infections, antibody deficiency.	XL	Xq28	36
9. Anhidrotic ectodermal dysplasia with (T-cell) immune deficiency (EDA-ID) *NFKBIA*	IKBα is an inhibitor of NF-κB activation. Anhydrotic ectodermal dysplasia with T-cell deficiency, mycobacterial and pyogenic infections.	AD	14q13	36
10. WHIM syndrome *CXCR4*	See E.1.j for details.	AD	2q21	40
11. GATA2 deficiency *GATA2*	Lack of dendritic cells, monocytes, B and NK lymphocytes (DCML). Susceptibility to mycobacteria (MonoMAC syndrome), papillomaviruses, histoplasmosis, lymphedema.	AD	3q21.3	Hsu AP et al. 2011;118:2653.

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
12. Pulmonary alveolar proteinosis (PAP) *CSF2RA*	Mutations in the colony-stimulating factor 2 receptor alpha (CSF2RA) impair GMCSF signaling, resulting in surfactant-derived lipoprotein accumulation in the lungs. Biallelic mutations within the X-Y pseudoautosomal region-1 (PAR-1).	AR	Xp22.33	41
13. Epidermodysplasia verruciformis				
a. Ever1 deficiency *TMC6*	Transmembrane channel-like 6 expressed in keratinocytes conferring resitance to HPV	AR	17q25.3	42
b. Ever2 deficiency *TMC8*	Transmembrane channel-like 8 expressed in keratinocytes conferring resitance to HPV	AR	17q25.3	42
G. *DNA Breakage-Associated and DNA Epigenetic Modification Syndromes* (*for Artemis, Ligase IV, and NHEJ1 deficiency, see A.2*)				
1. DNA breakage-associated syndromes				
a. Ataxia-telangiectasia (A-T) mutated (ATM) *ATM*	Cell cycle checkpoint ATM protein kinase	AR	11q22.3	47
b. Nijmegen breakage syndrome protein 1 (Nibrin) *NBS1*	Nibrin participates in DNA repair together with RAD50 and MRE11.	AR	8q21	48
c. Bloom syndrome *BLM*	DNA repair protein BLM	AR	15q26.1	48
d. A-T like disease (ATLD) *MRE11A*	DNA damage-response protein	AR	11q21	48
e. DNA ligase deficiency I *LIG1*	DNA ligase I is ATP-dependent.	AR	19	48
2. Immunodeficiency, centromere instability and facial abnormalities syndrome 1 (ICF1) *DNMT3B*	DNA (cytosine-5)-methyltransferase 3b	AR	20q11.2	49
3. Immunodeficiency, centromere instability and facial abnormalities syndrome 2 (ICF2) *ZBTB24*	Zinc finger and BTB domain-containing protein 24 (ZBTB24) is involved in DNA methylation. Same phenotype as ICF1.	AR	6q21	49
4. PMS2 deficiency *PMS2*	Class-switch recombination deficiency due to impaired mismatch repair resulting in CSR-induced DNA double-strand breaks in Ig switch regions. May present as hyper-IgM syndrome.	AR	7q22.1	27
5. RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features, learning difficulties) syndrome *RNF168* (Ring finger protein 168)	Defective DNA double-strand break repair	AR	3q29	Blundred M. Stewart GS. Expert Rev Clin Immunol 2011;7:169.
H. Defects of the Classical Complement Cascade Proteins				
1. C1q deficiency	SLE-like syndrome			
a. *C1QA*	Subcomponent A chain. C1qA chain.	AR	1p36.12	55
b. *C1QB*	C1qB chain	AR	1p36.12	55
c. *C1QC*	C1qC chain	AR	1p36.12	55
2. C1 deficiency				
a. C1r deficiency *C1R*	C1r subcomponent. Often combined with C1s defect.	AR	12p13	55
b. C1s deficiency *C1S*	C1s subcomponent. Often combined with C1r defect.	AR	12p13	55
3. C2 deficiency *C2*	*C2* gene is located within the MHC cluster. SLE, infections.	AR	6p21.3	55

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
4. C3 deficiency *C3*	Major factor for both classical and alternative complement pathways. Severe pyogenic infections, SLE.	AR	19p.13.3	55
5. C4 deficiency	SLE, infections			
a. *C4A*	C4A subunit deficiency	AR	6p21.3	55
b. *C4B*	C4B subunit. *C4A* and *C4B* are adjacent genes within the MHC cluster.	AR	6p21.3	55
6. C5 deficiency *C5*	C5 peptide. Initiates formation of the membrane attack complex (MAC). *Neisseria* infections.	AR	9q34.1	55
7. C6 deficiency *C6*	C6 peptide. Part of MAC. *Neisseria* infections.	AR	5p13	55
8. C7 deficiency *C7*	C7 peptide. Part of MAC. *Neisseria* infections.	AR	5p13	55
9. C8 deficiency (*Neisseria* infections)				
a. *C8A*	C8α-polypeptide	AR	1p32	55
b. *C8B*	C8β-polypeptide	AR	1p32	55
c. *C8G*	C8γ-polypeptide, binds covalently to the C8α-chain; C8 is part of MAC	AR	9q	55
10. C9 deficiency *C9*	C9 peptide. Part of MAC. *C6*, *C7*, and *C9* genes are clustered on chromosome 5p. *Neisseria* infections.	AR	5p13	55
	I. *Defects of the Alternative Complement Pathway*			
1. Factor B deficiency *CFB*	Factor B serine protease. Interacts with factor D. The gene is encoded within the MHC cluster.	AR	6p21.33	55
2. Factor D deficiency *CFD*	Factor D interacts with factor B.	AR	19p13.3	55
3. Factor H1 deficiency *CFH1*	Factor H deficiency leads to uncontrolled activation of the alternative C pathway. A polymorphism (Y402H) is responsible for ~50 percent of age-related macular degeneration.	AR	1q231.3	55
4. Properdin factor C deficiency *PFC*	Contributes to activation of C3 via the alternative pathway	XL	Xp11.23	55
	J. *Complement Regulatory Proteins*			
1. C1 inhibitor deficiency *C1NH*	C1 inhibitor, a serine protease inhibitor. Haploinsufficiency results in hereditary angioedema.	AD	11q12.1	55
2. C4-binding protein deficiency. Presumed defect in C4 binding; dissociates and degrades C4 (classical C pathway).				
a. *C4BPA*	C4 binding protein α	AR	1q32.2	55
b. *C4BPB*	C4 binding protein β	AR	1q32.2	55
3. Decay-accelerating factor (DAF) (CD55) deficiency *CD55*	Impairs C killing by controlling both pathways via inhibition of C3 convertase	AR	1q32.2	55
4. Factor I deficiency *CFI*	C3-inactivator	AR	4q25	55
5. CD59 (antigen P18-20) or protectin deficiency *CD59*	20 kDa GPI-anchored antigen. Inhibits lysis by classical C pathway.	AR	11p13	55

(continued)

Table 1.1 (CONTINUED)

DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
6. Mannose-binding lectin deficiency				
a. Mannose-binding lectin deficiency *MBL2*	Mannose-binding lectin activates a distinct, antibody-independent complement pathway.	AR and AD	10q21.1	55
b. Mannan-binding lectin serine protease 1 *MASP1*	Activates C3 and C2. Developmental facial syndrome; 3MC syndrome has been proposed as a unifying term encompassing the overlapping Carnevale, Mingarelli, Malpuech, and Michels syndromes.	AR	3q27.3	55
c. Collectin subfamily member 11 *COLEC11*	Developmental facial syndrome; 3MC syndrome has been proposed as a unifying term encompassing the overlapping Carnevale, Mingarelli, Malpuech, and Michels syndromes.	AR	2p25.3	55
d. Mannan-binding lectin serine protease 2 deficiency *MASP2*	Mannan-binding serine peptidase 2. Activates complement pathway via C4 and C2 by cleaving mannose-binding lectin.	AR	1p36..22	55
7. Miscellaneous complement defects				
a. Complement receptor 3 (CR3) deficiency, integrin, alpha M (ITGAM) *ITGAM*	*ITGAM* encodes the α-subunit of LFA-1 (Mac1), SLE.	AR	16p11.2	53
b. CD46 molecule *CD46*	Complement regulatory protein	AR	1q32	55
c. CD59 molecule *CD59*	Membrane attack complex inhibitor	AR	11p13	55
d. Ficolin deficiency *FCN3*	Ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen)	AR	1p36.11	55
e. Paroxysmal nocturnal hemoglobinuria *PIGA*		Acquired mutation (XL)	Xp22.2	55
	K. Periodic Fever Syndromes			
1. Familial Mediterranean fever (FMF) *MEFV*	The *MEFV* gene product, pyrin, controls ASC (apoptosis-associated speck-like protein)-induced IL1-caspase-1 cascade.	AR	16p13.3	33
2. Hyper-IgD with periodic fever syndrome *MVK*	Mevalonate kinase (MVK) regulates mevalonic acid and isoprenoid metabolism.	AR	12q24.11	33
3. Cold autoinflammatory syndrome due to cryopyrin deficiency. Mutations in NLR family pyrin domain containing 3 *NLRP3* (also called *CIAS1, NALP3* or *PYPAF1),* including:	Cryopyrin/Nacht domain-, leucine-rich repair and PYD-containing protein 3 (NALP3)/pyrin domain-containing Apaf1-like protein 1(PYPAF1) deficiency	AD	1q44	33
a. Familial cold autoinflammatory syndrome 1 (FCAS1)	Maculopapular rash, arthralgia, fever in response to cold			
b. Muckle-Wells syndrome (MWS)	Similar to FCAS1, progressive deafness, amyloidosis			
c. Chronic infantile neurological, cutaneous, and articular (CINCA) syndrome	Neonatal rash, chronic meningitis, anthropathy, recurrent fever			
4. Cold urticaria due to phospholipase Cγ2 (PLCγ2) deficiency *PLCG2*	Cold urticaria persistently present. Variable manifestations of atopy, granulomatous rash, autoimmune thyroiditis, ANA positive, sinopulmonary infections.	AD		Ombrello MJ et al. *N Engl J Med* 2012;366:330.
5. Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) *TNFRSF1A*	TNF receptor 1 (CD120a) mutations result in impaired TNF removal. Serositis, rash, arthritis.	AD	12p13.31	33

Table 1.1 (CONTINUED)

	DESIGNATION, GENE NAME*	DEFECTIVE PROTEIN, PATHOGENESIS	INHERITANCE	LOCUS	REFERENCE OR BOOK CHAPTER
6.	Blau syndrome due to defective nucleotide-binding oligomerization domain protein 2 NOD2 (also called CARD15) *NOD2*	Granulomatous polyarthritis, uveitis and cranial neuropathies; 30 percent develop Crohn's disease.	AD	16q12.1	33
7.	Early-onset inflammatory bowel disease				
	a. Due to IL-10Rα mutations *IL10RA*	IL-10 receptor, alpha	AR	11q23.3	33
	b. Due to IL-10Rβ mutations *IL10RB*	IL-10 receptor, beta	AR	21q22.11	33
	c. Due to IL-10 mutations *IL-10*		AR	1q32.1	33
8.	PAPA syndrome due to proline-serine-threonine phosphatase interacting protein 1 deficiency *PSTPIP1*	Pyogenic (sterile) arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome	AR	15q24.3	33
9.	Majeed syndrome lipin 2-deficiency *LPIN2*	Chronic recurrent multifocal osteomyelitis, congenital dyserythropoietic anemia, transient inflammatory dermatosis with neutrophil infiltration	AR	18p11.31	33
10.	Deficiency of the IL-1 receptor antagonist (DIRA) *IL1RN*	Skin and bone inflammation in the newborn period	AR	2q14.2	33

AD, autosomal dominant; AR, autosomal recessive; EBV, Epstein-Barr virus; NK, natural killer; XL, X-linked.

*Gene names, in italics, according to the Human Genome Organization, http://www.gene.ucl.ac.uk/nomenclature/; Wai Man, http://www.nebi.nlm.nih.gov/omim/.

CD4 T lymphocytes, and individuals with homozygous variants of this receptor have decreased HIV susceptibility. Thus, one could make the extreme argument that the majority of humans are immunodeficient with respect to HIV, while those lacking the CCR5 receptor constitute a rarer, resistant population. The dominantly inherited immune defect caused by the expression of fully functional CCR5 is manifested only upon HIV infection, with development over time of a generalized secondary immunodeficiency as HIV-infected cells are lysed and the T-lymphocyte count decreases. This example illustrates that the definition of what is actually a primary immunodeficiency is not always straightforward. Similar to many other PIDs, this CCR5 sufficiency "defect" can be treated by a transplant of hematopoietic stem cells from a donor lacking CCR5 expression (Hütter et al., 2009).

THE INCREASED RATE OF DISEASE GENE IDENTIFICATION AND MUTATION NUMBER

The number of identified genes and individual mutations causing PIDs is steadily increasing. With the unprecedented development of very high-throughput DNA sequencing technologies, it is likely that we will witness a truly dramatic increase in the diagnosis of PID over the next few years. Deep sequencing approaches to detect disease-causing mutations have already begun (Bolze et al., 2010; Puel et al., 2011). Historically, molecular disease mechanisms were first identified when an affected protein was recognized by coincidence. This was the case for adenosine deaminase (ADA) deficiency, discovered serendipitously in 1972 in an infant with SCID, who lacked the ADA protein (Chapter 13). Next followed the era when disease genes were mapped by linkage analysis in multiplex families, or in some instances by cytogenetic abnormalities, to certain chromosomal regions, whose content then needed to be ascertained. A causative gene and corresponding deleterious mutations were subsequently deciphered by painstaking molecular analysis. The first example of this approach was the discovery in 1986 of the *CYBB* gene underlying the X-linked form of chronic granulomatous disease. A patient with an intracellular oxidative bacterial killing defect as well as other phenotypes had a contiguous gene deletion including *CYBB* (Chapter 51).

X-linked immunodeficiency genes were among the first to be found because pedigree analysis restricted the proportion of the genome that needed to be tested and hemizygous males were affected; autosomal recessive genes, in contrast, were often found by studying consanguineous families in whom a disease gene could be sought in a limited region of DNA inherited from a common ancestor. Before the human genome was fully sequenced, identifying mutated genes by finding linkage regions was tedious. However, once a dense map of highly polymorphic markers and even more numerous single nucleotide polymorphisms (SNPs) was available, it became much easier to trace and identify disease-causing genes. Subsequently, having the complete human genome sequence and catalogs of known and predicted genes further facilitated PID gene discovery. Now new methods enabling high-throughput sequencing of targeted DNA regions or of

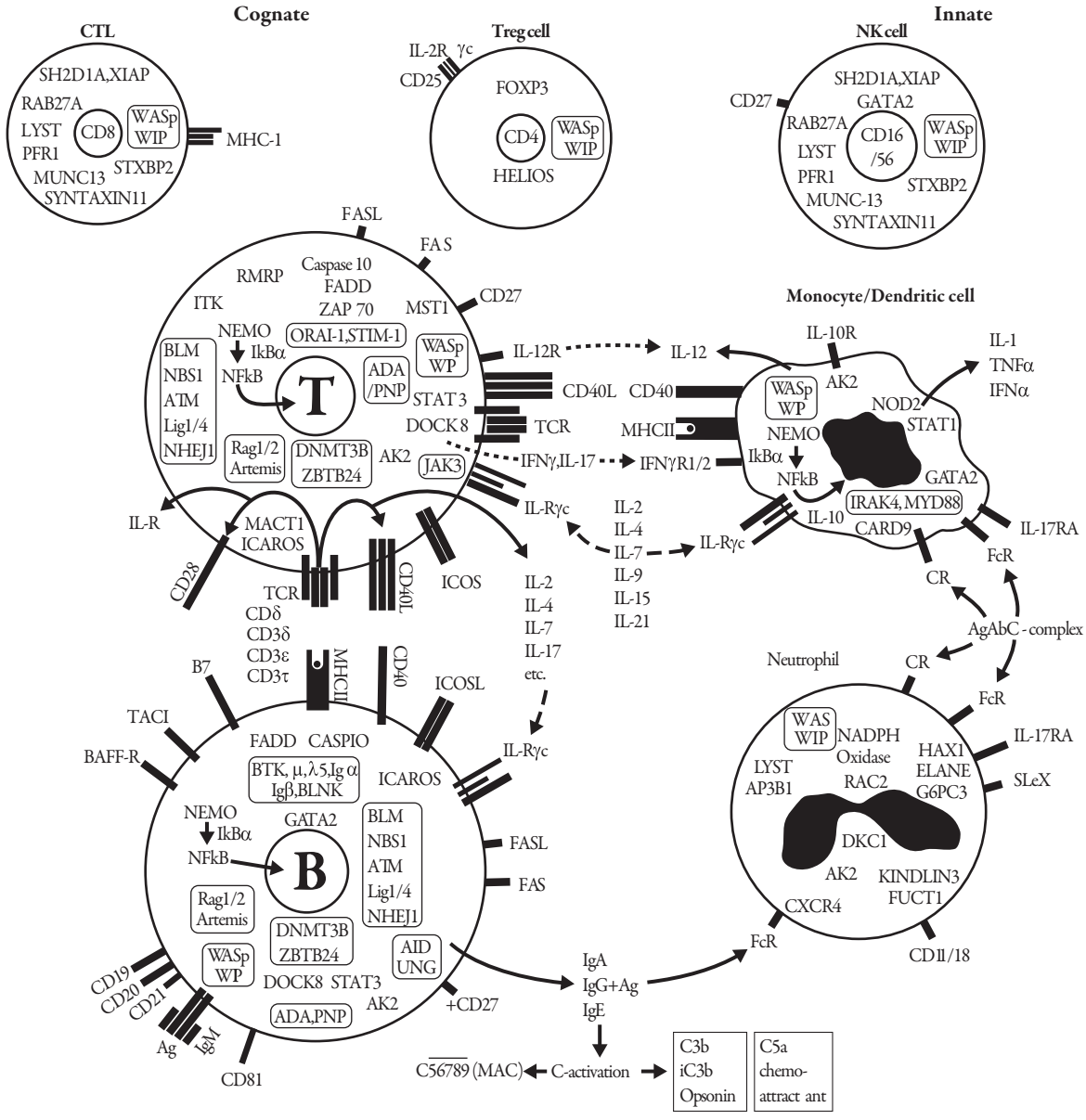

Figure 1.1 Simplified schematic diagram of mediators of leukocyte activation and interactions between T and B cells, macrophages, and neutrophils. IL-2Ryc [this lowercase y should be Greek symbol gamma], common chain of IL-2, 4, 7, 9, and 15 receptors; TCR, T cell receptor; SLeX, S-Lewis X receptor. For other abbreviations, see Table 1.1.

the entire exome (defined as the coding and immediate adjacent regions of genes) further assist direct searches for disease-associated sequence variants. With reduction of sequencing costs, detection of sequence variants throughout the entire genome will be increasingly affordable, although bioinformatics analysis remains a major challenge at present. In the future whole genome sequencing of newborns may identify many PIDs even prior to clinical manifestation. This will have a major impact on patient care.

For these reasons, future editions of this book are likely to contain many more disease genes, and individual gene mutations are already too numerous to catalog in print. In this volume there are so many new disease genes that the editors have decided to make some changes compared to the second edition. We have grouped related diseases into single chapters and introduced a new, short-chapter format for rare PID genes that do not belong to a functionally related group but rather form unique disease paradigms.

DISORDERS WITH FINDINGS OUTSIDE THE IMMUNE SYSTEM AS THE MAIN PRESENTATION

There are several multisystem disorders in which variable degrees of immunodeficiency may occur but are not the most severe or widely recognized feature. Although immune compromise may be severe in rare cases, it is more often mild or even inapparent. Such disorders include centromere instability syndromes (Chapter 48), ataxia-telangiectasia (Chapter 46), DiGeorge syndrome (Chapter 44) and cartilage-hair hypoplasia (Chapter 36). A recent addition is the hepatic veno-occlu-

sive disease with immunodeficiency described in Chapter 38. The reader is cautioned to consider the contribution of underlying immune pathology to both the frequent infections and the autoimmune phenomena that may accompany multisystem syndromes such as trisomy 21 and others beyond the scope of this book, for which the immune features are not predominant and malfunctions in other organ systems prevail.

MULTIPLE-MICROBE VERSUS SINGLE-MICROBE SUSCEPTIBILITY

Several excellent recent reviews have dealt with the distinction between patients in whom the infectious panorama involves multiple agents versus those whose susceptibility is limited to one or a few microbes (Alcaïs et al., 2009; Quintana-Murci et al., 2007). Traditionally, most PIDs belong to the group of multiple-microbe susceptibility. Thus, reticular dysgenesis and SCID patients are highly prone to most, if not all, infectious agents. However, retrospective analysis shows that when children born with such defects have succumbed to an infection, a limited number of pathogens have been implicated. This reflects common environmental exposure and lack of effective treatment, as well as the potential of certain agents such as adenovirus to cause lethal disease in a short period of time. Similarly, patients with less profound defects limited to B lymphocytes, complement or granulocytes, while being susceptible to a multitude of infectious agents, nevertheless show a more restricted infectious pattern than found in SCID. As expected, the more profound and global the immune defect, the wider is the range of infectious pathogens in the phenotype.

The other end of the multiple-microbe susceptibility spectrum can be exemplified by individuals with failure to make a specific immune system component. Those with low levels of IgG4 are not recognized to be particularly prone to infections, while only about one in three people lacking IgA, which is targeted to mucosal surfaces, show increased susceptibility to respiratory and gastrointestinal infections. However, even without an overt history of mucosal infections, those who lack IgA have increased susceptibility to infections caused by microbial species such as Giardia and also show an increased propensity for autoimmune disease (Chapter 27). These patients may demonstrate underlying disease mechanisms not directly related to their lack of IgA, as autoimmunity is generally uncommon in patients with XLA, who lack B lymphocytes and thus all classes of immunoglobulins (Chapter 24).

The above PIDs contrast strongly with the lymphoproliferative syndromes, disorders in which a single infectious agent, Epstein-Barr virus (EBV), is the cause of nearly all fatal outcomes (Chapter 43). Individuals with defects in the X-linked genes *SH2D1A* and *XIAP* or the autosomal genes *ITK* (Huck et al., 2009) and *CD27* (van Montfrans et al., 2012) may be completely healthy until exposed to EBV but develop life-threatening complications following infection with this virus. Thus, it appears that humans are endowed with several gene products that are essential for protection against lethal EBV infections. This is an important message, demonstrating the co-evolution of a human pathogen and host defenses. The proteins involved in the defense against EBV appear nonredundant, since there seems to be full penetrance of the defect in each genotype. Additional components of the innate immune system are postulated whose contribution to defense against severe EBV disease may be more nuanced or nonspecific. Such genes have yet to be identified.

EBV is not a unique example. Over the past decade hundreds of patients with defects in components involved in Th1 cytokine responses have been identified (Quintana-Murci et al., 2007; Chapter 34). In these disorders, infections due to mycobacteria, salmonella, and fungi are overrepresented. Thus, the lack of a particular immunocyte product results in a more selective phenotype as compared to the absence of an entire lymphocyte subpopulation. Surprisingly, results from experimental animal models suggest a defect that is more profound than the observed human infections, when the γ-interferon response is abrogated. These differences may be due to the large dose of infectious agents used in animal studies, as well as the route of administration, calling into question the relevance of these models for human disease.

Given the enormous number of microbial species, it seems quite possible that each and every one of us will have holes in the repertoire of humoral and cellular elements involved in protecting us against infections. To this end, a particular genetically determined variant of a single molecule could potentially protect us against one microbe while at the same time increase susceptibility to infection with another. The best example of such polymorphism is the major histocompatibility (MHC) locus, where certain HLA molecules efficiently present one antigen at the expense of another. Because each individual expresses only a limited number of MHC antigens, there must be important differences in how antigens are presented to T lymphocytes. In addition to HLA molecules, other components of the adaptive immune system not yet identified may be critical for determining susceptibility to individual infectious agents, but environmental factors such as age at exposure are also important.

CONTROLLING INFECTIONS: FROM ADAPTIVE TO INNATE IMMUNITY, TO FACTORS UNRELATED TO THE IMMUNE SYSTEM

Although anticipated, it became evident only recently that large numbers of autosomal recessive diseases exist, where the endogenous defense mechanism is mainly directed against single, or a few, infectious agents. A recent example is the increased susceptibility to herpes simplex virus (HSV), caused by mutations in *TLR3* encoding Toll-like receptor 3, *UNC-93B1* or *TRAF3*, encoding tumor necrosis factor receptor-associated factor 3 (Quintana-Murci et al., 2007, Perez de Diego et al., 2010, Chapter 35). The TLR3 defect causing susceptibility to HSV does not seem to primarily affect the adaptive immunity, but rather the innate immunity as manifested in cells of the central nervous system, where the mutation permits HSV to replicate and cause tissue damage. Very few affected individuals have been identified to date, and it appears that not all individuals carrying the mutation will develop severe HSV infection. The reason for this is not known.

The TLR3 defect resembles another disease, namely selective susceptibility to human papillomavirus (HPV), in that the affected cell does not belong to immunocytes. While TLR3 is still recognized as a representative of the innate immune system, the situation is different for epidermodysplasia verruciformis (Chapter 41) caused by mutations in the *EVER1* and *EVER2* genes, whose function is not known, but whose products have features of integral membrane proteins and are localized in the endoplasmic reticulum. It may well be that these proteins have no relationship to innate immunity.

Thus, one can easily imagine gene products whose primary functions are completely unrelated to innate or adaptive immunity, but when defective, clearly contribute to the propensity for infections. Whether such genes should be classified as immunodeficiency genes is debatable. At any rate, there will be many difficult future distinctions and definitions to be made, since certain gene products may remotely contribute to immune functions while other products may not.

NEW GENES FOR OLD SYNDROMES

Since the second edition was published, disease genes have been discovered for numerous long-recognized PID phenotypes. An example is chronic mucocutaneous candidiasis (CMM), for which Puel et al. (2011) have identified two disease mechanisms, both affecting interleukin-17. Mutations in the gene for IL-17F produce autosomal dominant disease, whereas defective interleukin-17 receptor A causes autosomal recessive disease. Importantly, the defective interleukin-17 signaling pathway results in susceptibility to infections with *Candida albicans* but not to infections with other pathogens. However, the most common cause for CMM seems to be gain-of-function mutations in the *STAT1* gene (Liu et al., 2011, van de Veerdonk, 2011). In this disease with autosomal dominant inheritance the increased STAT1-signaling seems to impair the induction of IL-17 immunity.

ACQUIRED SYNDROMES RESEMBLING PID

As mentioned above, ALPS can be caused not only by heterozygous germline *FAS* mutations but also by somatic ("acquired") mutations (Holzelova et. al, 2004) detected in the circulating double-negative T cells (CD3+CD4−CD8−, TCR αβ+). In a recent report, more than one third of ALPS patients had somatic mutations of the intracellular domain of *FAS*, resulting in loss of Fas-mediated apoptosis and selective survival (Dowdell et al., 2010). Somatic ALPS patients had elevated serum concentrations of vitamin B12, IL-10, and soluble Fas ligand, similar to ALPS patients with germline *FAS* mutations. A subset of ALPS (type IV) has been found to be caused by somatic mutations in RAS family genes (*NRAS*, *KRAS*); two unrelated infants with ALPS-like symptoms had the same heterozygous point mutation in the *KRAS* gene affecting hematopoietic cell lineages (Takagi et al., 2011).

Anticytokine autoantibodies have also emerged as a cause of susceptibility to infections. Neutralizing antibodies that target a particular cytokine known to participate in host defense against specific organisms have been observed in several unique patient populations: autoantibodies to granulocyte macrophage colony-stimulating factor (GM–CSF) cause pulmonary alveolar proteinosis (Chapter 40); anti-interferon (IFN)-γ autoantibodies have been associated with disseminated nontuberculous mycobacteria (Patel et al., 2005); and antibodies to IL-17A, IL-17F, and IL-22 have been found to explain the mucocutaneous candidiasis associated with autoimmune polyendocrinopathy, candidiasis, ectodermal dysplasia (APECED) syndrome (Puel et al., 2010; Kisand et al., 2010). Anti-IL-6 autoantibody was observed in a boy with severe staphylococcal cellulitis and subcutaneous abscesses (Puel et al., 2008). It is possible that these and other autoantibody-mediated infectious disease susceptibilities may be controlled with therapy to eliminate B cells.

REVERSION OF MUTATIONS CAUSING PID

Pathologic mutations that have reverted to wild type have been known previously but are now firmly established in several PIDs, including ADA-deficient, X-linked SCID and WAS. In these disorders, the spontaneous reversion or second-site mutations restored function and thus ensured a selective survival or growth advantage for the corrected cells (Liu et al., 2009; Stephan et al., 1996; Wada et al., 2001). Less obvious are reversion mutations in CD8 T cells of three patients with leukocyte adhesion deficiency type I (LAD-1) reported by Uzel et al. (2008). It is unclear if these CD8+CD18+ T lymphocytes were functionally active.

PROGRESS IN DIAGNOSIS AND TREATMENT

Progress in immunology, genetics, and molecular biology has changed the way we diagnose and treat PID patients. This is illustrated by the histories of families with multiple generations of affected members. Figure 1.2 represents a kindred now known to carry XLA (Chapter 25). Inspection of the pedigree reveals that individual III-3, born in 1918, was an obligate carrier of this condition. It is interesting that she had two brothers who died early in life; although definitive information is lacking, they may have been affected with XLA. Her oldest son, IV-3, suffered from recurrent upper and lower respiratory tract infections and died of pneumonia in 1948 at the age of 4 years. Her two younger sons, IV-4 and IV-6, were able to receive the newly available antibiotic penicillin. They survived multiple episodes of pneumonia and were diagnosed with XLA in the mid-1950s, shortly after Bruton's discovery of agammaglobulinemia (Bruton,

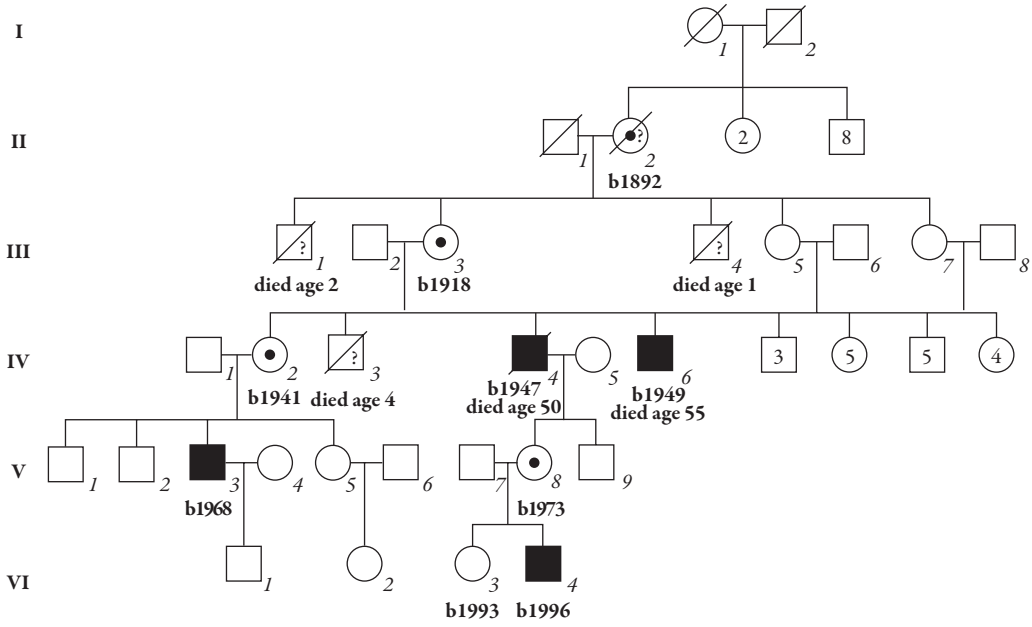

Figure 1.2 Pedigree of a family with X-linked agammaglobulinemia (XLA), illustrating medical progress in diagnosing and treating affected males. Males, squares; males diagnosed with XLA, filled squares. Females, circles; mutation carriers, circles with filled center. Slash, deceased. Individuals who may have had the XLA mutation are indicated with a question mark. Year of birth (b) and age of death are indicated for selected individuals. Individuals III-1, III-4, and IV-3 died of pneumonia. IV-4 died of pulmonary insufficiency, having suffered many bouts of pneumonia from early childhood until diagnosis at age 7.

1952). By the time intramuscular immunoglobulin treatment was instituted, both boys had developed chronic lung disease and bronchiectasis. IV-4 was one of the first patients treated with high doses of intravenous immunoglobulin (IVIG) for echovirus infection with dermatomyositis, fasciitis, and meningitis (Mease et al., 1981). He died of chronic respiratory failure at the age of 50; his younger brother (IV-6) died at the age of 55.

The younger generations of this family have a much more hopeful prognosis. When individual V-3 developed his first pneumonia at the age of 2, he was referred to a university center, where the diagnosis of XLA was confirmed and treatment with immunoglobulin initiated. He has remained healthy with regular immunoglobulin replacement, now by subcutaneous self-infusion, and at present has a full-time job and no chronic disease. XLA was diagnosed in the youngest member of this kindred, VI-3, at the time of birth by documentation of absent B cells in cord blood and sequence analysis of *BTK*. He was immediately started on immunoglobulin treatment and has remained completely healthy.

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2.

GENETIC PRINCIPLES AND TECHNOLOGIES IN THE STUDY OF IMMUNE DISORDERS

Jennifer M. Puck and Robert L. Nussbaum

INTRODUCTION

Medical genetics is concerned with the investigation, diagnosis, treatment, counseling, and management of patients and families with inherited disease. Research in medical genetics focuses on identifying the genes involved in human hereditary diseases and the changes in DNA sequence that cause or predispose to these diseases; elucidating disease pathogenesis, including both genetic and environmental factors; understanding the inheritance patterns of diseases in families; developing new treatments or cures for hereditary disorders; and helping patients and families make reproductive decisions and cope with the impact of genetic disorders on the health of family members. Although a complete discussion of medical genetics is beyond the scope of this book and can be reviewed in several texts (Nussbaum et al., 2007; Scriver et al., 2001; Speicher et al., 2010), we present here an overview of the terminology, concepts, and methods of molecular genetics. This introduction will outline the approaches that have led to the identification of the genes involved in over 200 specific disorders of the immune system. Newer and more powerful methods of deep "next-generation" sequencing and genomic analysis will make possible further revolutionary changes, not only in the discovery of additional disease genes, but also in understanding the pathogenesis of defects in host defenses. Using this understanding has led to and will continue to lead to effective new therapies.

The normal complement of 46 chromosomes in a human cell consists of 23 pairs of homologous chromosomes (Fig. 2.1). Twenty-two of the 23 pairs are *autosomes* and are the same in men and women; the remaining pair, the *sex chromosomes*, consist of two X chromosomes in females or one X and one Y (carrying the male-determining genes) in males. The two homologous chromosomes that make up each of the 22 pairs (23 pairs in females) are identical in size, centromere placement, and arrangement of genes.

The location of a particular gene on a chromosome is known as the *locus* for that gene. The DNA sequence in and around a gene (such as in introns or flanking regions or even within coding regions) may vary among the individuals in the population, so the *reference sequence* of the human genome is defined as our best approximation of frequently found sequences in a large collection of individuals (http://genome.ucsc.edu; Fujita et al., 2011). The term *allele* is used to describe the DNA sequence at a locus, which may be identical to or different from the reference sequence, and the particular pair of alleles an individual possesses for a given gene is called his or her *genotype*. Alleles can be rare, deleterious mutations that cause disease or frequent normal variants of no known significance. If greater than 1 percent of the alleles in the population have a DNA sequence at a locus that differs from the reference DNA sequence, the locus is said to be polymorphic. A polymorphism can be a single nucleotide change (*single nucleotide polymorphism* or *SNP*), a small deletion or insertion of a few nucleotides (*indel*), a difference in the number of repeated of di-, tri-, or tetra-nucleotide units contained in a short tandem repeat (*STR*), or a deletion or duplication of segments containing hundreds to millions of nucleotides, known as a *copy number variant* (*CNV*). A group of polymorphic alleles at a set of loci close together in a row on a single chromosome is called a *haplotype*. When the alleles in a particular haplotype are found to remain together over many generations, the alleles are said to be in *linkage disequilibrium* (LD). LD is a measurable quantity and ranges from 0 (no LD) to 1 (complete LD).

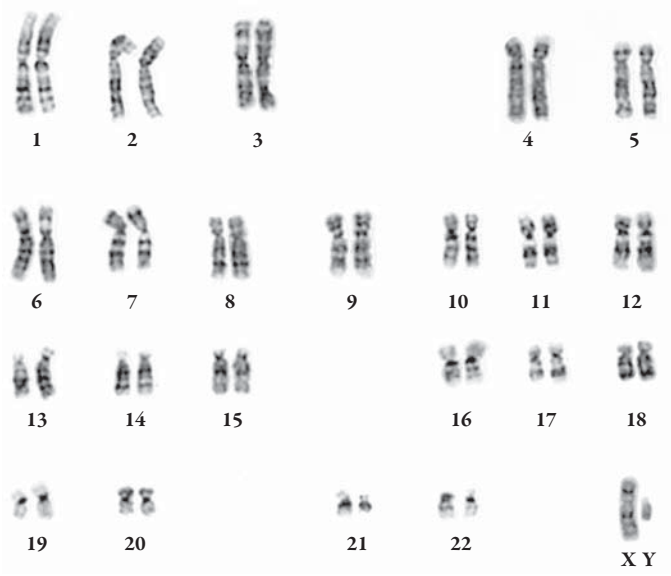

Figure 2.1 Normal human metaphase chromosomes from a male, aligned to show the 22 pairs of autosomes, one X, and one Y. The banding pattern is revealed by Giemsa staining. Giemsa-dark regions are particularly rich in genes. (Kindly provided by Amalia Dutra.)

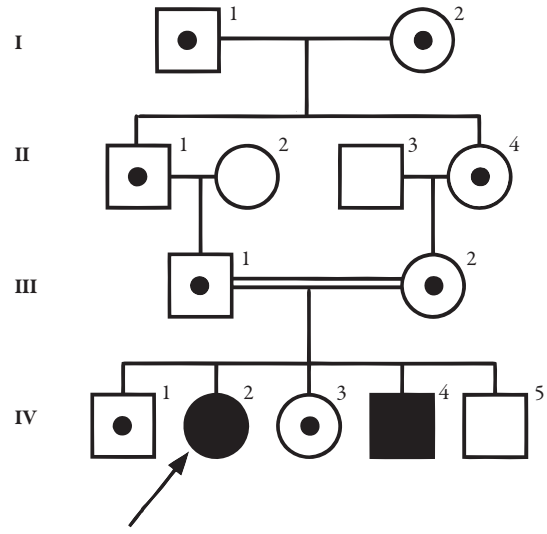

Figure 2.2 Autosomal recessive inheritance. Circles, females; squares, males; filled symbol, affected; symbol with dot, silent carrier. Roman numerals designate generations, with each individual within a generation identified by an integer. This pedigree demonstrates a consanguineous mating, double horizontal line, between cousins III-1 and III-2 (a typical but not required characteristic of autosomal recessive disease pedigrees). An arrow marks the proband, the first person in the family to come to medical attention. For updated conventions for pedigree drawing, see Bennett et al. (1995).

MECHANISMS OF INHERITANCE

MENDELIAN VERSUS COMPLEX INHERITANCE

Mendelian inheritance describes the hereditary patterns in diseases caused by DNA mutations of big effect in single genes inherited from parents by their offspring. Thus, Mendelian disorders include most of the rare primary immunodeficiency diseases described in this book. An increasing awareness of a genetic contribution to other diseases has led to the designation *non-Mendelian* or *complex inheritance* to refer to instances in which an individual's genetic makeup has a more variable and more complex role in disease causation. Examples of complex diseases include multifactorial diseases in which one or more gene mutations must interact with each other and/or environmental factors in order for a disease to be manifest.

AUTOSOMAL RECESSIVE INHERITANCE

In autosomal recessive inheritance (Fig. 2.2), disease usually results when a person inherits two defective copies of the same autosomal gene; the affected individual can be either a homozygote, if two identically defective copies of the same gene are inherited, or a compound heterozygote, if each gene copy has a different deleterious mutation. The parents are unaffected heterozygotes, who carry one normal copy and one abnormal copy of the gene. By definition, heterozygous carriers of recessive disorders are not affected because the normal copy of the gene compensates for the defective copy. The typical familial inheritance pattern in autosomal recessive illness is for disease to occur in one or more full brothers and sisters, with both genders affected equally and both parents unaffected. Recessive disorders are seen with increased frequency in children of consanguineous marriages, in which the parents are related to each other (Fig. 2.2), share ancestry, or come from genetically isolated populations. The underlying mutations carried by the parents are presumed to have arisen in a single common founder. The genotypes of affected individuals in such instances are expected to show homozygous mutations. A lack of a family history of affected relatives is not an argument against autosomal recessive inheritance because many contemporary families have small sibship sizes, making a single affected child born to unaffected parents the rule rather than the exception.

Recessive illness usually results from loss of function of a gene whose product is normally present in excess, so that even a half-normal amount of the gene product is adequate to prevent disease. For example, homozygotes with deletion of the first exon of the gene encoding adenosine deaminase (ADA) have a profound deficiency of the enzyme, resulting in early onset of severe combined immunodeficiency (SCID) (see Chapter 13). In contrast, heterozygotes, with one deleted and one functional copy of the gene, have an amount of enzyme activity in between that found in normals and that in patients with ADA-deficient SCID; however, even a reduced amount of the normal enzyme is sufficient to remove purines toxic to lymphocytes and thus protect a heterozygous individual from expressing any immunological defect.

AUTOSOMAL DOMINANT INHERITANCE

In pure autosomal dominant inheritance (Fig. 2.3), an individual needs only one copy of a gene alteration for disease to occur, and homozygotes for the alteration have the same severity of disease as do heterozygotes. The parent from whom the genetic alteration was inherited may himself or herself be affected or may be a silent, or *nonpenetrant*, carrier. In humans,

very few dominantly inherited disorders are truly dominant. Instead, they are usually incompletely dominant, which means an individual heterozygous for the mutation is affected but homozygotes for the mutation are far more severely affected. The typical inheritance pattern in autosomal dominant illness is to see multiple affected individuals, both genders affected equally, with transmission of the disease from one generation to the next.

If a dominantly inherited condition requires the presence of an alteration on only one chromosome, it would seem contradictory that some individuals whose position in the pedigree implies they must be heterozygotes for the disease-causing mutation (*obligate heterozygotes*) show no evidence of the disease (lack of penetrance), as illustrated for individual II-4 in Figure 2.3. However, such lack of penetrance is a well-described phenomenon in autosomal dominant disorders. It is seen if the onset of the disorder is age-dependent, or if an additional factor or factors, such as a second spontaneous somatic mutation or an environmental agent or influence, must be superimposed on the underlying genetic defect in order for the disease to become clinically evident. An example of an autosomal dominant primary immune disorder is autoimmune lymphoproliferative disease (ALPS, see below and Chapter 30).

A lack of family history is not a strong argument against autosomal dominant inheritance because a nonpenetrant carrier parent makes the affected individual appear as a sporadic case of the disease in the family. Alternatively, it is possible that neither parent harbors the alteration if the child's mutation arose spontaneously after separation of the germline of one of the parents from somatic cell progenitors, a situation known as *germline mosaicism*. With germline mosaicism, a parent can make gametes carrying the mutation and pass the mutation one or more times to a child without the germline mosaic parent demonstrating the mutation in the somatic tissues (blood, buccal cells, fibroblasts) that are readily available for mutation testing. New mutations may also occur in an embryo after conception and may be present in the germline but also in some, but not all, somatic tissues. In this situation, known as *somatic mosaicism*, the mutation in the child may or may not be detectable in somatic tissues available for mutation testing.

The rate at which new mutations occur per gamete was previously estimated by measuring the incidence of completely penetrant autosomal dominant or X-linked diseases, such as achondroplasia or Duchenne muscular dystrophy, in a large cohort of newborns. The measured rate of gene mutations per gamete that resulted in a detectable clinical phenotype ranged over nearly two orders of magnitude, from 10^{-6} to 10^{-4}. With the availability of whole-genome sequencing, we now have an unbiased estimate of mutation rate of 1.1×10^{-8} per base (95 percent confidence interval, 0.68 to 1.7×10^{-8}), or approximately 33 new mutations among the ~3 billion base pairs of DNA in a gamete.

Mutations can cause dominantly inherited diseases through a number of different mechanisms. In the most straightforward situation, abnormal amounts of the gene product may be inadequate. For example, deficiency of one copy of a gene contained within microdeletions of chromosome 22 can be responsible for DiGeorge syndrome (see Chapter 45). This model for dominant inheritance is called *haploinsufficiency*. In contrast, more than two copies of a gene may also cause disease inherited in an autosomal dominant manner, as is seen with duplication of the *PMP22* gene in Charcot-Marie-Tooth IA peripheral neuropathy (Boerkoel et al., 2002) or triplication of the α-synuclein gene in familial Parkinson's disease (Singleton et al., 2003).

A second pathogenetic mechanism for dominant genetic disease is a *gain of novel function*, in which a new or altered protein produced from the mutated allele is endowed with a novel or toxic activity not found in the normal gene product. An example is multiple endocrine adenomatosis type 2, in which activating mutations in the *RET* oncogene lead to medullary thyroid carcinoma, hyperparathyroidism, and pheochromocytoma.

A mechanism for a single mutation causing dominant disease is *dominant negative* mutation, as is seen in most patients with ALPS (see Chapter 30). Patients with heterozygous mutations in the gene encoding the apoptosis mediator CD95/Fas have defective programmed cell death, normally initiated through the homotrimeric Fas receptor complex. In vitro studies show not only failure of the mutant protein to transmit a death signal itself, but also interference by mutant Fas molecules when associated with normal Fas molecules in the death-inducing signal complex (Fisher et al., 1995). The mutated Fas proteins produce steric interference when incorporated into the normal trimeric Fas receptor complex.

Finally, in some dominantly inherited cancer syndromes there is a *two-hit* mechanism (Knudson, 1971). The first hit is a mutation inherited through the germ line that inactivates one allele of a *tumor suppressor gene*, thus rendering an individual heterozygous for the mutated gene. One defective allele, however, is not sufficient to cause disease until a single somatic cell undergoes a second event inactivating the remaining normal allele. After this second hit, the cell may undergo

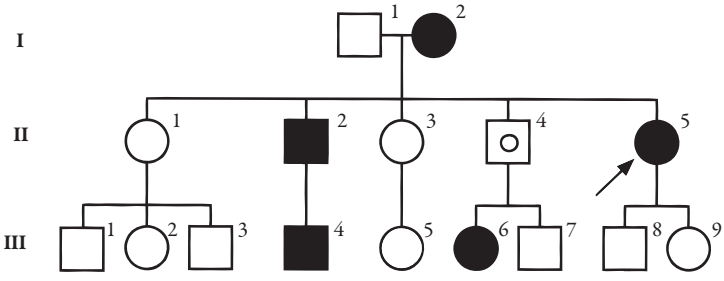

Figure 2.3 Autosomal dominant inheritance. Note male-to-male transmission from subject II-2 to III-4, strongly suggesting dominant inheritance. II-4 is a nonpenetrant carrier. Circles, females; squares, males; filled symbol, affected; symbol with circle, at risk but currently unaffected carrier; arrow, proband.

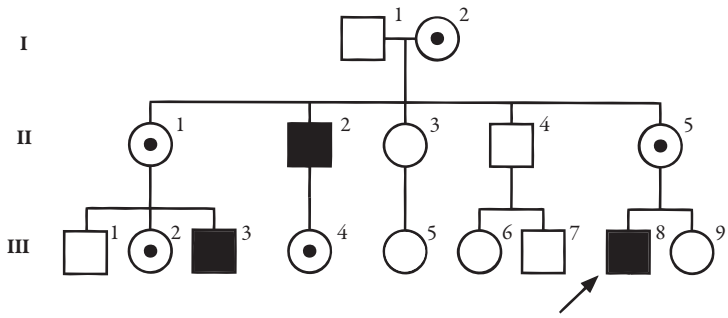

Figure 2.4 X-linked inheritance. Circles, females; squares, males; filled symbol, affected; symbol with dot, silent carrier. Male-to-male transmission not present.

pathological, unregulated growth to produce a clonal cancer. Second hits may be large genomic deletions in a single cell as it divides, resulting in *loss of heterozygosity* for SNPs in the vicinity of the disease locus in the tumor cells. On the other hand, the second hit may be an epigenetic change (such as abnormal DNA methylation) of the remaining normal allele, resulting in inactivation without any alteration of the primary nucleotide sequence. Accumulation of multiple somatic hits after the initial two hits increases pathogenic features of many cancers. The Lynch syndrome of hereditary colon and endometrial cancer caused by mutations in genes responsible for DNA mismatch repair is a classic example of an autosomal dominant syndrome that satisfies a two-hit model.

X-LINKED INHERITANCE

Mutations in X-linked genes have strikingly different consequences in males and females and cause diseases with a distinctive X-linked pattern of inheritance (Fig. 2.4). Males have only one copy of genes on their single X chromosome, while women have two. To provide dosage compensation and equalize gene expression for X-linked genes in males and females, one of the two X chromosomes in a female's somatic cells is chosen at random, early in embryonic life, to undergo a near-total and irreversible inactivation (Lyon, 1966). In a male carrying a defective X-linked gene, clinically apparent abnormality always occurs because his only copy of the gene is disrupted. Male *hemizygosity* for the X chromosome explains the large number of X-linked immunodeficiencies (Fig. 2.5) and the high proportion of males diagnosed with inherited immunodeficiency.

In females with X-linked gene defects, the situation is more complex. In contrast to the situation with a heterozygote for an autosomal gene mutation, a female heterozygous for an X-linked gene mutation does not have a uniform population of cells, each of which expresses both the normal and abnormal gene. Instead, the somatic tissues of a female are made of a mixture of cells, some of which have an active X chromosome carrying the normal gene, while the rest have an active X chromosome carrying the abnormal gene. The relative proportion of cells with one or the other X chromosome active in any one tissue averages 50 percent but may differ substantially depending on chance and the number of precursor cells for that tissue that were present in the embryo when X inactivation took place. The fraction of cells in a tissue that have an active X chromosome carrying the normal gene is usually sufficient for normal function of the tissue, so female heterozygotes for X-linked disorders are usually silent carriers. If, however, the cells that have inactivated the X chromosome carrying the normal gene predominate in a tissue, a female heterozygote may be symptomatic. Thus, the terms "dominant" and "recessive" as applied to autosomal disorders are not strictly applicable to X-linked conditions.

In female carriers of some X-linked diseases, such as X-linked SCID due to defects in the IL-2 receptor γ chain (see Chapter 10) or X-linked agammaglobulinemia (XLA) due to defects in the Bruton (or B-cell) tyrosine kinase Btk (see Chapter 25), the expected random X inactivation in the lymphocyte population targeted by the gene defect is not seen (Puck, 1993). In these situations, a female carrier of X-linked

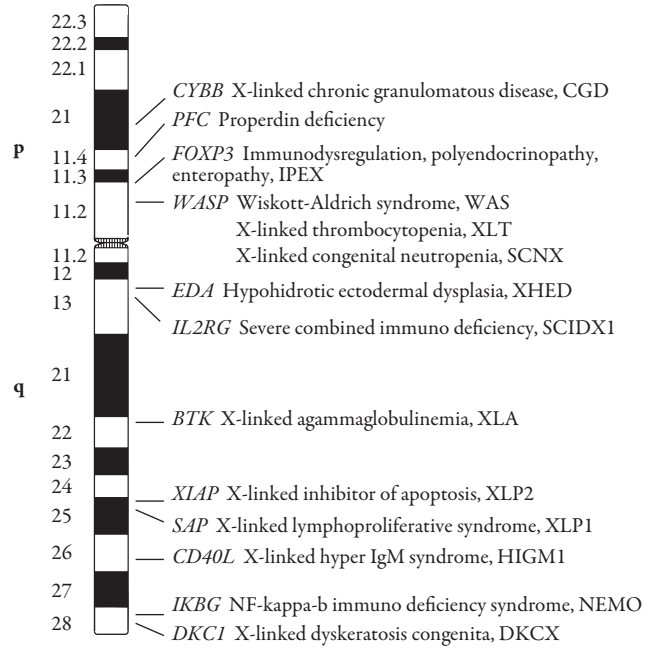

Figure 2.5 Idiogram of human chromosome X, illustrating the major Giemsa bands and conventional cytogenetic nomenclature, including p (short) arm and q (long) arm, integers denoting major bands increasing from centromere to telomere, and additional digits to the right of the decimal point denoting sub-bands. The loci on the X chromosome of 12 known human immune disease genes are indicated.

SCID (or of XLA) will have no lymphocytes in the case of SCID (or no B cells in XLA) whose active X chromosome carries the mutation because the fraction of the lymphocyte precursors that have inactivated the X chromosome carrying the normal version of the gene cannot develop and survive normally. As a result, the target lymphocyte population for each disease will show marked "skewing" of X inactivation. The abnormal X-inactivation pattern seen in X-linked immunodeficiencies has been useful for carrier identification in the past but has largely been replaced by direct mutation assessment.

A typical X-linked inheritance pattern (Fig. 2.4) is characterized by affected male siblings and cousins as well as affected males in additional generations, all affected members of the kindred being related through unaffected female relatives. However, a lack of family history does not rule out an X-linked inheritance mechanism for immune deficiency. As with autosomal dominant disorders, a spontaneous new mutation in an X-linked gene can result in somatic or germline mosaicism for a mutation and cause the disease to appear in the family, either directly in a male or by the creation of a silent female carrier who passes the mutation on to her sons. One third of the cases of X-linked diseases severe enough to prevent reproduction by affected males are expected to be the first manifestation in their family of a new mutation (Haldane, 1935). Because males donate a Y chromosome and not an X chromosome to their sons, documenting male-to-male transmission of any trait in any pedigree rules out X-linked inheritance.

HETEROGENEITY

Genetic heterogeneity is a broad term used to describe departures from the simple models of "one gene—one enzyme" or "one mutation—one disease." Diseases can show *allelic heterogeneity*, in which different mutations in the same gene cause disease, as is the rule in single-gene immunodeficiency diseases. *Locus heterogeneity* occurs in diseases such as SCID in which a similar phenotype of absent cellular and humoral immunity leading to opportunistic infections can arise from mutations in a number of different genes; the diseases caused by mutations at these different loci are termed *genocopies*. In contrast, a *phenocopy* is an acquired, not a genetic, disease that resembles genetic forms of the disease.

Still more complicated models of inheritance appear to be operating in disorders such as common variable immune deficiency and IgA deficiency (see Chapter 28) or in atopic disease. In these disorders one can observe a clearly increased incidence within families; however, an obvious Mendelian pattern of inheritance is absent. A genetic contribution to such diseases is suspected when there is greater concordance of the disease in monozygotic (identical) twins as compared to dizygotic (fraternal) twins, and when there is an increased risk for the disease in relatives of affected individuals as compared to the population at large. The risk of a second affected person in a family is greater the closer the blood relationship with the proband. Such complex inheritance patterns, well described in asthma and insulin-dependent diabetes mellitus, are the result of interactions between genes at different loci combined with unidentified but substantial environmental effects.

HOW TO IDENTIFY DISEASE GENE LOCI

ABNORMAL PROTEIN PRODUCTS

The first disease genes to be recognized as mutated in immune disorders were identified by defining abnormalities in their protein products. ADA was originally identified as a purine metabolic enzyme, and subsequently the absence of ADA activity was noted to occur in patients who lacked lymphocytes and had immune defects (see Chapter 14; Giblett et al., 1972). This approach was fruitful in ADA-deficient SCID because the enzyme encoded by the disease gene turned out to be a "housekeeping" gene, expressed in all normal cells. The general method of first identifying protein abnormalities in immune disorders and then documenting gene lesions has been less successful for disease genes that have restricted tissue expression or are active only in an early stage of differentiation of the target cell type. For these, positional cloning and identifying mutations in candidate genes have been essential.

CYTOGENETIC ABNORMALITIES

When clinical disorders are associated with abnormalities in the number or structure of an individual's chromosome complement, cytogenetic techniques can lead to the identification of disease genes. Metaphase chromosomes from dividing cells can be stained with Giemsa dye to reveal segmental banding patterns that uniquely characterize each human chromosome (Fig. 2.1). Cytogenetic analysis involves comparison of each chromosome to the standard karyotype idiogram, as illustrated for the X chromosome in Figure 2.5, and can identify translocations and show deletions or duplications large enough to be visible under the microscope (>2 to 5 Mb, depending on location). Hybridization of labeled DNA probes to denatured chromosomes, known as fluorescent in situ hybridization (FISH; Plate 2.I), can pinpoint much smaller regions of the genome to indicate the chromosomal location of a disease-associated microdeletion, duplication, or rearrangement. Comparative genome hybridization (CGH) is a powerful newer technique that reveals much smaller, cytogenetically invisible deletions or duplications. In CGH, the patient's DNA, labeled with a fluorescent dye that emits at a "green" wavelength, is mixed in equal proportions with a standard DNA labeled with a dye that emits in the "red" spectrum. The mixture is then denatured and hybridized to an array of hundreds of thousands of oligonucleotides corresponding to DNA sequences throughout the genome. If the number of copies of DNA in the patient and reference sam-

ples containing a particular nucleotide sequence are equal, the spot on the array corresponding to that sequence will fluoresce yellow, representing equal contributions of DNA labeled with green and red. If the patient has a deletion of a segment, the DNA in the mixture representing that segment will have less green and more red and the spot will fluoresce more toward the red; if the patient has a duplication of a segment, that spot will demonstrate excess fluoresce in the green part of the spectrum. The resolution of array CGH depends on the density and loci of the oligonucleotides on the array. While perfectly balanced translocations are not detected by CGH, this technique has become the preferred way, rather than karyotype analysis or FISH, to diagnose copy number variations such as the interstitial deletions in DiGeorge syndrome.

Aneuploidy, or abnormal chromosome number, is associated with immune defects. Trisomy 21, or Down syndrome, the most common genetic cause of human mental retardation, is accompanied by depressed in vitro immune responses and by an increased incidence of autoimmunity and infections, the leading cause of death in Down syndrome (Epstein, 2001; Ram and Chinen, 2011). In addition, some infants with trisomy 21 have sufficiently low T cell numbers to be flagged as abnormal in population-based newborn screening for SCID (Kwan et al, 2013).

At the subchromosomal level, *contiguous gene deletion syndromes* are collections of consistently observed clinical manifestations resulting from deletions spanning multiple neighboring genes. An example is the chromosome 11p13 deletion syndrome associated with Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (Haber, 2001; Schaffer et al, 2001). Larger deletions produce the complete phenotype, while smaller disruptions involving single genes within the region produce limited phenotypes, such as isolated aniridia.

Rare or unique cytogenetic abnormalities are occasionally found in the context of genetic disorders. Coexistence of a chromosomal translocation, duplication, or deletion with an abnormal phenotype in a patient is unlikely to be a coincidence; rather, the cytogenetic lesion may provide evidence for the genetic localization of a disease. One of the best examples is the contiguous gene deletion syndrome produced by interstitial deletion of chromosome Xp21 in male patients suffering from multiple disorders, including chronic granulomatous disease as well as Duchenne muscular dystrophy, retinitis pigmentosa, and McLeod hemolytic anemia (see Chapter 52; Schaffer et al, 2001).

DISEASE GENE LOCALIZATION BY LINKAGE MAPPING

DNA Polymorphisms

Each child inherits one of each pair of homologous chromosomes from one parent and the other chromosome of the homologous pair from the other parent. The parent of origin of each chromosome can be identified by tracing the inheritance of different polymorphic alleles. The most common polymorphisms are SNPs, which occur approximately every 1,000 base pairs and are therefore useful not only for linkage but also for association studies (see below).

Meiotic Crossing Over

In the absence of abnormal cytogenetic findings to point to the chromosomal locus for a genetic disorder, a disease gene may be mapped by *linkage analysis* in kindreds in which the disorder affects multiple individuals. Linkage analysis relies on the normal phenomenon of *meiotic recombination*, or crossing over during gametogenesis. During the first meiotic division, each pair of homologous chromosomes lines up randomly on the spindle and then separates in the course of the first reduction division of meiosis I. This independent assortment of chromosomes during meiosis I is responsible for randomly distributing one member of each pair of homologous chromosomes into each gamete. It is also in meiosis I that homologous segments of two chromatids form a pair of homologous chromosomes that interchange their genetic material by crossing over at points of contact, known as chiasmata (Fig. 2.6). On average, between two and four chiasmata develop between every pair of homologous chromosomes during each meiosis.

Suppose two polymorphic loci are situated at locus 1 and locus 2 on the same chromosome, as shown in Figure 2.6, and there are polymorphic alleles "A" and "a" at locus 1 and alleles "B" and "b" at locus 2. Also suppose that a parent is heterozygous at both loci (genotype Aa Bb), and, in addition, allele A at locus 1 happens to be on the same chromosome (same DNA molecule) as allele B at locus 2, while alleles a and b are both on the other chromosome. If no crossing over occurs in

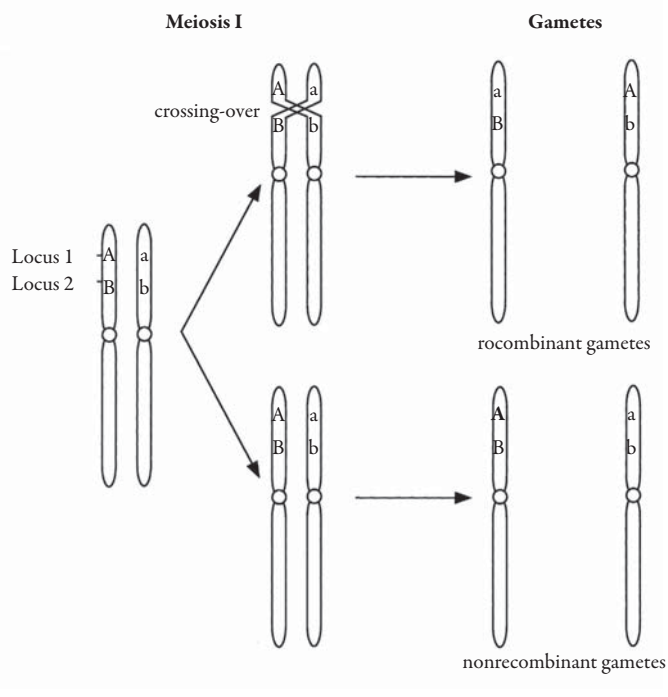

Figure 2.6 Diagram of meiotic crossing over involving two hypothetical loci, 1 and 2. Alleles at locus 1 are A and a; alleles at locus 2 are B and b. Recombinant gametes are products of a crossover event between locus 1 and locus 2.

the interval between locus 1 and locus 2 during meiosis, each gamete will receive either the chromosome containing alleles A and B or the one carrying alleles a and b (*nonrecombinant*). If, however, crossing over occurs between the two loci in the Aa/Bb individual, the resulting gametes will have a chromosome with a new (*recombinant*) combination of alleles, either alleles Ab or aB. From a knowledge of the genotypes of parents and their offspring, one can count the number of offspring resulting from a gamete carrying a crossover between locus 1 and locus 2 and determine the observed *recombination frequency* with which crossing-over events happen between the two loci during gametogenesis.

Because crossing over occurs approximately uniformly along a chromosome, the chance of a crossing-over event between two loci reflects how far apart they are on that chromosome. The further apart the loci are physically, the greater the chance that at least one crossover will occur between them; if they are very close to each other, the chance of crossing over will be small. If two loci are so far apart that at least one crossover will always occur in the chromosomal interval between locus 1 and 2 during gamete formation, then 50 percent of all the offspring will have the nonrecombinant and 50 percent the recombinant genotype. At the other extreme, when two loci are so close together that crossovers almost never occur, the observed recombination frequency will approach zero. Between these two extremes loci are *linked* on a chromosome. For such loci, the frequency of recombinant offspring will be between 0 percent and 50 percent, and one can correlate recombination frequency with the actual distance between two loci. When two loci have a recombination frequency less than 10 percent, recombination frequency translates directly into a theoretical *genetic distance*, in units called centiMorgans (cM), where 1 percent recombination is equal to 1 cM. This relationship does not hold when loci are loosely linked (recombination frequencies >10 to 20 percent) because of the chance that two independent crossovers, rather than just one, will occur between the two loci. A double crossover will not be detected as a recombination between the two loosely linked markers and, thus, the measured recombination frequency will always be less than the genetic distance in cM. In physical terms, the average recombination rate across the entire genome is 1.2 cM per megabase of DNA, which means that 1 cM of genetic distance holds around 830,000 base pairs of DNA (Kong et al., 2002).

Model-Based Linkage Analysis

Linkage analysis is used to map genes responsible for diseases that are inherited in a classical Mendelian pattern (Borecki and Suarez, 2001). Affected and unaffected members of families in which the disease is being inherited are studied by determining their genotypes at a large number of polymorphic genetic marker loci whose positions are known along each chromosome. Linkage analysis depends on looking in families for co-inheritance of a disease allele and alleles at polymorphic loci located in the chromosomal region near the disease gene. Recombination events between the disease locus and all of the genetic markers are tabulated. If the inheritance pattern is known by inspection of the pedigree (model-based analysis), one can score each affected and unaffected individual as either showing or not showing a crossover between the disease locus and each marker locus tested to determine the recombination frequency. Most markers will show a 50 percent recombination frequency because they are not linked to the disease locus, indeed not even on the same chromosome as the disease locus. If a marker seems to show less than a 50 percent frequency of offspring carrying chromosomes with a recombination between the marker locus and the disease locus, this marker may be linked to the disease locus. Model-based linkage works best when one or a few loci of reasonably high penetrance are responsible for a disease and, therefore, demonstrate a Mendelian inheritance pattern in families.

The statistical method used to measure the recombination frequency between genetic loci is called the maximum logarithm-of-odds (*LOD*) score method, and its features are summarized in Table 2.1. The result of a LOD score analysis consists of two parts. The first part, called θ_{max}, is the best estimate (in a statistical sense) of the recombination frequency between the disease locus and a polymorphic marker locus in a set of families. The value of θ_{max} is, therefore, a measure of genetic distance between two loci. The second parameter, Z_{max}, is a measure of how good that estimate of θ_{max} actually is. When Z_{max} is greater than 1.5, linkage is strongly suggested. If Z_{max} is larger than 3, the likelihood that the loci are linked is a thousand times (10^3) greater than the likelihood that the linkage data are purely the result of chance. Thus, LOD scores with Z_{max} of 3 or greater are taken as nearly definitive proof that two loci are linked.

LOD score analysis will demonstrate which polymorphic loci of known location are linked to the disease by finding the smallest value of θ_{max} with the largest Z_{max}, preferably 3 or greater. Linkage of the disease gene to a marker of known location on a chromosome thereby places the disease gene in the same general location as the marker on that chromosome.

ASSOCIATION STUDIES

In contrast to linkage analysis, association studies are usually done in populations, not families (Borecki and Suarez, 2001).

Table 2.1 **CHARACTERISTICS OF THE LOD SCORE**

1. The LOD score is a measure of the degree of linkage between two genetic markers.
2. The LOD score information comes in two parts: θ**, the recombination fraction** ***z*****, the logarithm of the odds that the markers are linked rather than unlinked**
3. θ **measures how frequently recombination occurs between two genetic markers. It is a statistical average derived from observing how often a recombination is seen in actual families.** ***The smaller*** θ ***is, the closer two markers are to each other.***
4. z is a logarithmic measure of how good the estimate of θ **really is. Every integer increase in z is a 10-fold improvement in certainty that** θ **"really" is what it has been measured to be.** ***The higher the z, the more significant is the measurement of*** θ***.***

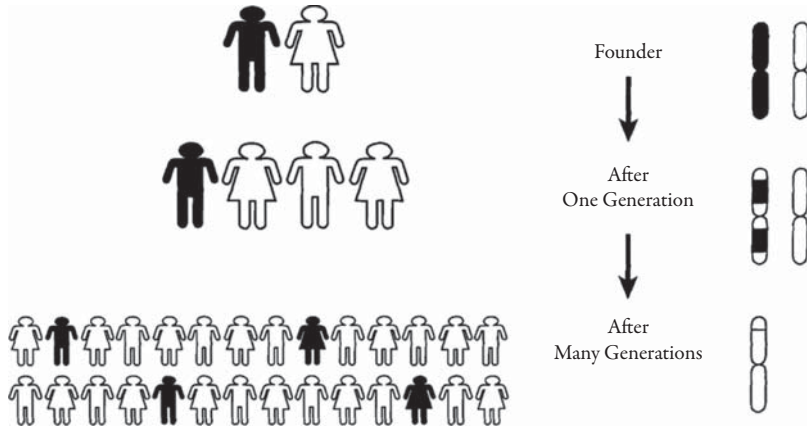

Figure 2.7 Linkage by descent in a hypothetical population in which a disease mutation can be traced to a founder. Marker alleles very close to the disease gene are unlikely to be separated by crossing over and constitute a disease-associated haplotype, which is preserved over many generations. (Kindly provided by Dennis Drayna.)

Association studies are particularly useful for common diseases with complex inheritance, as opposed to rarer diseases with Mendelian inheritance. In diseases with complex inheritance, the genetic contributions to a disease are subtle and involve alleles at many different loci, as well as environmental influences, that increase disease susceptibility. As a result, we see an increase in disease prevalence in close relatives compared to the general population because relatives tend to share more of the disease-susceptibility alleles than do unrelated population controls. Another characteristic of diseases with complex inheritance is that when one identical (monozygotic) twin has the disease, the other twin, who shares all of the disease susceptibility alleles with the affected twin, is more likely to develop the disease than would a dizygotic twin who shares only half the alleles. In diseases with complex inheritance, a Mendelian inheritance pattern is absent and standard linkage analysis cannot be applied. Instead, association studies can be used to find the genetic variants responsible for increasing disease susceptibility.

Suppose a disease-susceptibility allele arose many generations ago (Fig. 2.7), in a region of a chromosome with a particular haplotype defined by a particular set of alleles at nearby loci (Todd, 2001). An association between the disease-susceptibility allele and this haplotype is likely to be preserved through many generations because the loci containing the alleles that define the haplotype are so close to the disease locus that they are unlikely to recombine during the meioses that occurred as the disease gene was passed down through the generations; thus, the disease and haplotype will be in linkage disequilibrium, which means that the haplotype on which the disease allele originated will be found with increased frequency among affected individuals as compared to the population in general. Such increased frequency can be assessed using standard epidemiological tools such as case-control, cross-sectional, or longitudinal cohort studies.

In cross-sectional or cohort studies, in which all the affected individuals in a population are ascertained, an under- or over-representation of a genetic variant (an allele, a single-locus genotype, or a haplotype involving multiple loci) among affected individuals is measured using the *relative risk ratio* (RR ratio) in a two-by-two table (Table 2.2). The RR ratio is calculated as

$$\frac{\frac{a}{(a+b)}}{\frac{c}{(c+d)}}$$

i.e., RR is the *fraction* of individuals with a given genotype who have the disease divided by the *fraction* of individuals without that genotype who have the disease. A RR ratio of more than 1 indicates an association between the genotype and the disease in the population, while a ratio less than 1 suggests the genotype is protective. The significance of any deviation from 1 must of course be assessed by an appropriate statistical test.

In case-control studies, the relative risk has no meaning because the number of case and controls are chosen arbitrarily, independent of the prevalence of the condition in the population. For case-control studies, the *odds ratio* (OR) is calculated instead from a two-by-two table. The OR is the *odds* that an individual with a particular genotype has the disease versus the *odds* that an individual without the genotype has the disease:

Table 2.2 **RELATIVE RISK RATIO ANALYSIS**

The relative risk (RR) ratio describes the risk of having a disease with versus without a given genetic determinant:

	DISEASE PRESENT	DISEASE ABSENT
Haplotype present	**a**	**B**
Haplotype absent	**c**	**D**

RR ratio = [a/(a + b)]/[c/(c + d)].

$$\frac{\frac{a}{(b)}}{\frac{c}{(d)}} = ad/bc$$

Note that when the disease is rare (i.e., a<b and c<<d), the RR ≈ OR.

Association studies can be powerful tools for identifying alleles that appear to increase or decrease susceptibility to various diseases. Suppose, however, we do not know the relevant variants and genes for which we wish to test for association and want to assess association between a disease and *every* genetic variant throughout the genome. This approach, known as a genome-wide association study (GWAS), has been heavily exploited over the past 10 years, due primarily to the identification of many millions of variants resulting from the haplotype mapping (HapMap) project. This project, the second major effort of the Human Genome Project to be undertaken once the first sequence of a human genome was completed, revealed $\sim 8 \times 10^6$ alleles as of 2010. The alleles discovered through the HapMap project have been used to define haplotypes that are in linkage disequilibrium (LD blocks). Finding an association with any of the alleles within an LD block means it is likely that some allele(s) within the LD block, either known or unknown, are responsible for the association.

Association studies must be interpreted with care (Nussbaum, 2013). First, poor study design or inadequate numbers of cases and controls can lead to false-positive or false-negative results. Second, even when a study has been done properly and with appropriate quality-control standards, an association by itself does not prove a causal relationship between the variant alleles and the disease phenotype. All alleles in linkage disequilibrium with a variant found to be associated with a disease will also show an association without being directly, causally related, although documentation of linkage of a risk factor to all such alleles (a risk-associated haplotype) would provide important information. For example, the iron-overload disease hemochromatosis and certain alleles at the HLA-A locus are closely associated, but the HLA-A alleles themselves do not actually cause hemochromatosis. Instead, there is significant linkage disequilibrium between particular HLA-A alleles and mutations at the closely linked *HFE* gene that is actually responsible for the disease (Gandon et al., 1996).

The variants in the HapMap project are for the most part polymorphic variants, meaning they are fairly common, occurring in more than 1 percent of alleles in at least one, and usually most, human populations. Thus, using the HapMap variants to do genome-wide association assumes that complex diseases will have their major genetic contributions from relatively common alleles, an assumption referred to as the "common disease—common variant" hypothesis. This hypothesis has now been tested in hundreds of common diseases with complex inheritance, with mixed results. In some cases, such as in macular degeneration or Crohn's disease, variants have been found in genes that are functionally related to the disease process and are responsible for a substantial fraction of the genetic susceptibility to these diseases. In most cases, however, such as coronary artery disease or type 2 diabetes mellitus, the genetic contribution of common alleles to complex diseases is very modest, with relative risks or odds ratios ranging from just over 1 up to 2. Furthermore, the fraction of the total genetic susceptibility conferred by these alleles is quite small.

Table 2.3 INTERNET RESOURCES FOR GENETIC INFORMATION

NAME	INTERNET ADDRESS	DESCRIPTION
NCBI	www.ncbi.nlm.nih.gov	National Center for Biotechnology Information, a division of the National Library of Medicine. Repository for genomic sequence, cloned genes, expressed sequence tags, Unigene project data, and more.
UCSC Genome Bio-informatics	http://genome.ucsc.edu/	University of California, Santa Cruz. Annotated assembly of human and other genomes, including genes, predicted genes, markers, and more.
Ensembl Genome Browser	http://www.ensembl.org/	The Wellcome Trust, Sanger Institute genomic information site. Repository for genomic sequence, comparative species homologies, genes, predictions, and more.
OMIM	http://OMIM.org	Online Mendelian Inheritance in Man, an online, searchable "knowledge base" of human genetic disorders, including clinical description, genetic data, and molecular characterization of known disease genes
GeneTests	http://www.genetests.org/	Publicly funded medical genetics information resource listing clinical and research laboratories providing diagnostic testing for genetic diseases in North America
Immune Deficiency Foundation	http://www.primaryimmune.org/contact.asp	U.S. national organization dedicated to research, education, and advocacy for primary immune deficiency diseases
Jeffrey Modell Foundation	http://www.jmfworld.com/ or http://npi.jmfworld.org/win/index.cfm	U.S. and international immunodeficiency network
European Society for Immunodeficiencies	http://www.esid.org/	Physician information, disease databases

POSITIONAL CLONING

The availability of the entire sequence of the human genome has revolutionized disease gene identification once linkage or association to a specific genetic region has been established. The entire complement of genes in any region of human DNA is now readily ascertained by searching public databases containing the complete human genome sequence deposited by the Human Genome Project (Baxevanis, 2001), and all the genes located in this region can be evaluated as candidates for alterations responsible for the disease phenotype. In addition to genomic sequences, mRNA sequences from all the expressed genes in a wide variety of tissues are also available (Riggins and Strausberg, 2001) (Table 2.3).

SCREENING OF CANDIDATE GENES

Once the set of candidate genes in the critical region of linkage or association is obtained through mining genomic DNA and mRNA sequence databases, candidates can be evaluated to determine which one is likely to be the disease gene. Northern blots or reverse transcriptase PCR (rt-PCR) help to determine the tissue expression pattern and to test for alterations in mRNA size or quantity in patients affected with the disease. Ultimately, sequence comparison must reveal mutations in the alleles of affected patients that are not present in the alleles of healthy controls, and studies of mutant gene function may be required to prove that the mutations are deleterious.

The changes in DNA that constitute deleterious mutations in genes are extremely heterogeneous. In some cases, deletion of part or all of the DNA encoding the gene has occurred, resulting in production of no mRNA or a defective mRNA transcript lacking important portions of the gene sequence. Small insertions or deletions of a few base pairs may shift the triplet reading frame and create mRNA carrying incorrect codons and, often, a premature termination downstream of the small insertion or deletion. In other cases, single point mutations can interfere with normal RNA processing, such as splicing of exons, resulting in mRNAs that either retain noncoding intron sequences in the transcript or lack all or part of a coding exon. Some point mutations within coding regions change an amino acid codon into a stop codon (*nonsense mutation*), thereby inserting a premature termination into the mRNA. Still other point mutations may alter a codon from that of one amino acid to another (*missense mutation*). Regardless of which of the above mutation mechanisms has occurred, mutant mRNAs are frequently poorly transcribed or highly unstable; cells carrying such mutations may appear to lack mRNA entirely rather than producing abnormal mRNA.

Mutations that occur over and over again on independent genetic backgrounds are known as *hot-spot mutations*. The most frequent is the alteration of the dinucleotide cytosine-guanine (CG) to thymidine-guanine (TG) either on the coding or anticoding strand; this particular mutation occurs approximately 11 times more frequently than any other single-base mutation in the genome. It is believed that the cytosine within a CG dinucleotide is at high risk for undergoing C-to-T transition because such cytosines are frequently covalently modified by methylation at the 5 position of the pyrimidine ring. Spontaneous deamination of a methylcytosine results in a thymidine, which may not be repaired promptly by the cell because it is not recognized as an abnormal base within DNA. Hypotheses for the mechanisms of these mutations and data on their frequency, as well as a lexicon of mutations observed in human genetic disease, were first compiled by Cooper and Krawczak (1995).

The impact of any particular mutation depends on the effect of that mutation on the transcription of the gene into mRNA, the translation of the mRNA into a protein product, and the post-translational processing and stability of any mutant protein that is made. Moreover, the functional consequences of mutations also depend on the physiological and environmental context of the cell, tissue, and individual carrying the mutation. In many diseases, a myriad of different mutations can lead to the same final common pathway: the loss of a functional gene product. In the inherited immune disorders as a rule, there are dozens or hundreds of different mutations that can disrupt function of the gene product and bring about the disease. However, in certain types of genetic disease, a particular mutation may affect the function of a protein in a subtle and unique way and thereby cause a highly specific phenotype. For example, only a few, highly specific missense mutations in the β-globin gene result in a protein with the physicochemical properties that cause the hemoglobin to form crystals under low oxygen conditions, as seen in sickle cell anemia. Thus, the mutation repertoire for sickle cell disease is severely restricted. Similarly, a restricted type of mutation in the Wiskott-Aldrich protein gene causes neutropenia rather than the typical phenotype of thrombocytopenia (see Chapter 43).

By means of positional cloning, human disease genes have been identified solely on the basis of their genomic location with little or no knowledge of the nature of the pathophysiological process responsible for the disease. These mutations overwhelmingly affect the coding portions of genes or occur at highly conserved regions of introns immediately adjacent to exons. The coding portions of genes, however, constitute only 4 to 5 percent of the sequence of a gene, and thus constitute a mere 1 to 2 percent of the genome. With detailed information on the sequence of the human genome, we are discovering further alterations in noncoding regions that contribute to disease. In such cases, linkage mapping or association studies are only the beginning of the study of pathogenesis of genetic disease and correlations between genotype and phenotype. Such variants may be in 5' or 3' regulatory sequences or introns and may alter expression or function only to a limited degree. Variants affecting levels of gene expression are not always adjoining the coding sequence; examples are known of locus control regions, enhancers, and other sequences altering expression that are 10 to 100 or more kilobases distant from the coding sequences they influence. There are also small interfering RNA (siRNA) molecules encoded throughout the genome that recognize and target specific transcripts for degradation and an increasingly appreciated array of epigenetic modifications of gene expression. Finding such mechanisms and proving they contribute to human disease is a major challenge to current and future genetics research.

After the completion of the first human genome sequences in 2003, predictions of great improvements in technology

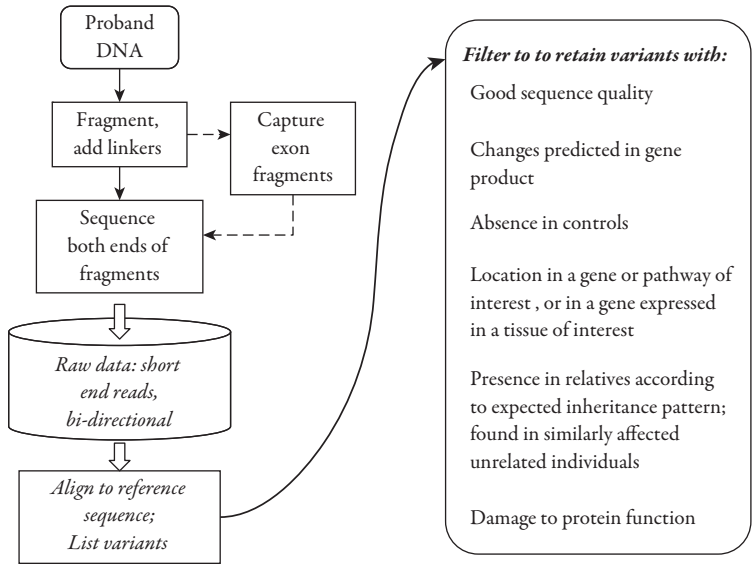

Figure 2.8 Schema for whole-exome sequencing. Input genomic DNA is fragmented, linkers are attached that carry signatures similar to barcodes for tracking, and hybridization to a fixed set of exome reference oligonucleotides is carried out with the remaining DNA washed away. After exon capture, the DNA is submitted to high-throughput sequencing, and short sequence reads are assembled and mapped to the human genome. Variants from the reference genome can then be analyzed.

with corresponding reduction in cost were made, including that a full genome sequence for any individual would eventually be obtainable for merely $1,000. The era of deep sequencing has arrived even ahead of predictions, with many reports of disease-gene identification through sequencing all of the coding DNA (whole exome) or even the whole genome of affected individuals (Bamshad et al., 2011; Bick and Dimmock, 2011; Cirulli and Goldstein, 2011; Teer and Mullikin, 2010). Methodologies are rapidly evolving, but Figure 2.8 shows how whole-exome or whole-genome sequencing and analysis are done, starting with fragmentation of genomic DNA, selective capture of sequences containing exons and immediate surrounding DNA (for exome sequencing), and generation of short "reads" of sequence, each around 100 bp, by high-throughput methodology. Next, the short sequences are aligned to the reference human genome, removing nonmatching sequences as presumed artifacts. Variants that do not match the reference sequence are listed, and this list can be subjected to further filtering, as shown in Figure 2.8, to remove nonsynonymous changes and common population variants from consideration, such as those in the dbSNP database. Further prioritization is based on absence in control individuals, tissue expression or involvement in a particular pathway or network (if known), and hypothesized mode of inheritance. Generation of whole-genome sequencing may become more prevalent, and even routine, but efficient analysis still represents a major challenge.

ETHICAL ISSUES IN GENETIC TESTING

There are numerous ethical issues surrounding human genetics as scientific knowledge and medical technology come to grips with the four central tenets of medical ethics: *beneficence* (doing good for the patient), *nonmaleficence* (doing no harm), *autonomy* (safeguarding the individual's rights to control his or her medical care and be free of coercion), and *equity* (ensuring that all individuals are treated equally and fairly). A full discussion of these issues is beyond the scope of this chapter, but a few examples arising in immunodeficiency diseases serve to illustrate some of these aspects of medical ethics and genetics.

As more and more genes are identified and found to be involved in human genetic disease, our ability to diagnose them will continue to increase dramatically. An inescapable consequence of this rapidly accelerating knowledge is the time lag between acquiring the ability to diagnose genetic disease and developing effective interventions to prevent diseases or treat them once they are diagnosed. For the immunodeficiency diseases, recent advances in therapy, from new antibiotics to immunoglobulin replacement to bone marrow transplantation and gene therapy, have placed clinical immunologists at the forefront of developing the tools to close the gap between diagnosis and treatment. How such tools are used, and whether they are readily available to all who need them, is clearly an issue of fairness in the application of scientific knowledge.

The ability to perform population-based newborn screening for SCID and other conditions with low T-lymphocyte levels has raised the possibility of making preinfectious diagnosis, but at this writing not all states in the United States have committed to adding this testing to their newborn screening panels, nor is the optimal treatment for newborns with SCID established (see Chapters 9, 59, and 60).

An additional aspect of the growth of genetic knowledge is in the area of disease predisposition. Increasingly, whole-exome sequencing and whole-genome sequencing allow for identification of individuals and their relatives who are at risk for disease that may have an onset much later in life than when the testing is being done. Testing healthy individuals for disease predispo-

sitions encoded in their genomes has obvious benefits in identifying people at risk who may be able to modify their lifestyles and/or begin appropriate preventive therapy, but it also carries the risk of serious adverse psychological damage and stigmatization in society. Discrimination in insurance and employment was also a serious issue, but the passage of laws such as the Genetic Information Nondiscrimination Act (GINA) in the United States and similar laws in other countries has reduced, but not eliminated, these concerns. Ethical problems with genetic testing are especially acute when the testing identifies a predisposition for a disorder for which current medical technology provides little or no treatment when clinical disease actually develops. The issue of beneficence becomes central in this setting. Is knowing that one carries such a gene doing more good than harm, or more harm than good?

The ethics of genetic testing is also an issue when testing children for the carrier state of diseases, such as X-linked immunodeficiencies, that pose no threat to a female child's own health but identify a substantial risk for having affected offspring. The autonomy of children, including their right to make decisions for themselves about learning about their own genetic constitution, must be balanced against the desire of parents to obtain such information and transmit it to their children in the manner they believe best. As the amount of available genetic information increases, there is an increasingly greater need to educate health-care providers, patients, their families, and society at large to make informed decisions about using genetic information wisely and for maximum benefit.

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3.

MAMMALIAN HEMATOPOIETIC DEVELOPMENT AND FUNCTION

Gerald J. Spangrude

The origins of the mammalian immune response are found in hematopoiesis. The primary immunodeficiency diseases discussed in this book are mediated by mutations in genes that function during the course of hematopoietic development at the level of the progenitors for specific cell lineages (severe combined immunodeficiencies), or at the level of the expression of signaling molecules that promote differentiation (major histocompatibility complex class II deficiency), or at the level of the functional potential of mature effector cells (leukocyte adhesion deficiency, chronic granulomatous disease). The common theme underlying the primary immunodeficiency diseases is the origin of the cellular components of specific and nonspecific immunity, the hematopoietic stem cell (HSC). While specific mutations in particular genes can result in defective function of highly specialized mature cell populations, the resulting immunodeficiencies are distinguished by the biochemical result of the disrupting mutation manifested at a stage of development much later than the HSC. This presents a unique opportunity in the design of HSC replacement therapies for some of these diseases.

Bone marrow transplantation (today mainly in the form of mobilized HSCs collected from the blood) is one avenue of HSC replacement therapy for a number of immunodeficiencies, as discussed in Chapter 60. Complications due to graft-versus-host disease often mean that in the absence of a related, genetically matched donor, the risks inherent in allotransplantation can outweigh the potential benefits. An exception to this generalization is severe combined immunodeficiency (SCID), which is 100 percent fatal without marrow transplantation (excluding adenosine deaminase and purine nucleoside phosphorylase deficiencies, which can be treated by enzyme replacement therapy). Transplantation of gene-corrected autologous cells can overcome the allotransplantation issue, as demonstrated by gene therapy trials for a variety of immunodeficiencies discussed in Chapter 61. While a number of immunodeficiencies can be corrected by gene therapy, it is important to recognize that inserting and inducing expression of an exogenous gene in order to correct a developmental defect may be accompanied by an equally disruptive alteration in hematopoietic function as a result of expressing the corrected gene in cell lineages where it is not normally found. Further, insertional mutagenesis is a valid concern, as shown by the recent experience in gene therapy for X-linked SCID. These obstacles to effective gene therapy for primary immunodeficiency diseases are the focus of current research in vector design, regulation of gene expression, and targeted insertion of genetic elements into the genome.

This chapter will provide an overview of our current understanding of hematopoiesis, specifically focusing on the transplantable HSC, to provide a basis for considering the common origin of primary immunodeficiency diseases and the prospects for bone marrow transplantation and gene therapy as therapeutic interventions.

OVERVIEW OF HEMATOPOIETIC STEM CELLS

Because of the relatively short lifespan of most blood cells, a high rate of production is necessary throughout life to maintain normal numbers. In adult mammals, the major source of blood is the bone marrow. It is here that the HSC is found, represented at a frequency of less than 1 cell per 10^4 marrow cells (Neben et al., 1993).

The HSC is now known to represent a heterogeneous mixture of cells with various degrees of functional activity in transplant and culture assays (Kiel et al., 2005). The identification and physical isolation of discrete stages of hematopoietic development has provided insight into the hierarchical nature of the hematopoietic lineages and has generated controversy regarding the developmental potential of cells at specific stages of development (Ceredig et al., 2009). Fundamentally, the HSC compartment can be considered as comprising two components, a dormant population and a proliferating population (Fig. 3.1). Various lines of experimental evidence indicate that these primitive HSCs enter the cell cycle only infrequently (Bradford et al., 1997, Cheshier et al., 1999), and they are generally thought of as being metabolically quiescent (Hodgson and Bradley, 1984). These conclusions were based on in vivo labeling studies using bromodeoxyuridine (BrdU), a nucleoside analog that is incorporated into DNA during cell division and can subsequently be detected by immunostaining. Cells that retain the BrdU label for a prolonged period of time after a pulse labeling period are termed "label-retaining cells," and their presence indicates cell cycle dormancy. However, truly dormant cells would not be expected to incorporate the BrdU label unless the pulse labeling time is extended to encompass the rare cell divisions that occur during the dormant state. A recent study has demonstrated that BrdU actually induces proliferation of HSC, likely by inducing a mild genotoxic injury sufficient to promote DNA repair (Wilson et al., 2008). Using a unique approach of inducible fluorescent labeling of histone proteins, Wilson et al. were able to characterize the dormant and activated subsets of HSC in the absence of proliferation induced by the labeling method. These experiments demonstrate that a two-way interconversion between the dormant and activated states occurs depending upon hematopoietic demand (Fig. 3.1). Cells making up the dormant subset replicate roughly once every 145 days in the mouse, suggesting that these cells do not contribute to ongoing hematopoiesis. However, this small subset of cells, estimated to be about 600 cells per mouse or about 1 per 2×10^4 bone marrow cells, includes the most potent cells for long-term reconstitution following transplantation into irradiated recipients. The HSC compartment is maintained to ensure lifelong blood production through a process known as *self-renewal*, defined as cell division cycles to replace HSCs that are lost due to differentiation into the multipotent progenitor compartment (Fig. 3.1). The pool of expanding multipotent progenitors retains developmental potential for all hematopoietic lineages but has largely lost the ability to self-renew. After an undefined number of cell divisions, these cells differentiate as various types of mature cells, depending on the cytokines available in the specific areas of the marrow where they mature. Recent studies utilizing prospective isolation of progenitor cell populations have demonstrated that one of the initial lineage specification events in normal hematopoiesis is the separation of the megakaryocyte and erythroid lineages from the myeloid and lymphoid lineages (Adolfsson et al., 2005). However, whether this segregation of lineages is absolute remains controversial (Forsberg et al., 2006), and the subsequent cellular intermediates in the pathways of differentiation that lead to the mature blood cells continue to be debated (Ceredig et al., 2009).

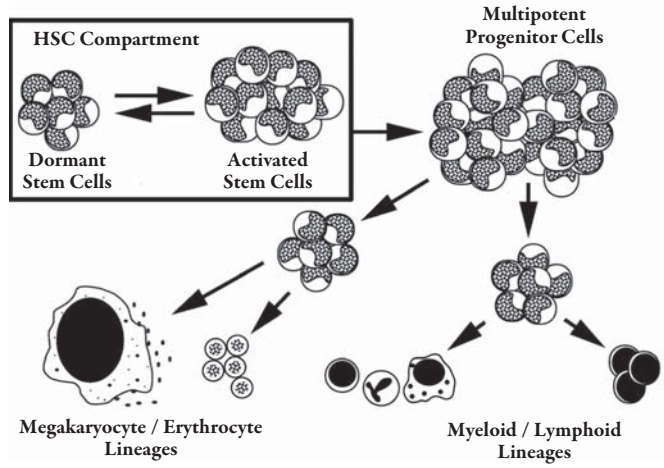

Figure 3.1 A model of the hierarchy of primitive hematopoietic stem cells (HSCs) and progenitor cells. Dormant stem cells are representative of the pluripotent HSCs, with both self-renewing potential and the ability to differentiate as any type of blood cell. These HSCs occasionally enter the cell cycle and replicate, during which time they are considered activated. Activated stem cells can return to dormancy (Wilson et al., 2008), effectively self-renewing the dormant pool, or may continue to proliferate and differentiate into the expanding multipotent progenitor cell pool, with a concomitant loss of the potential to self-renew. The expanding multipotent pool can differentiate as lymphoid or myeloerythroid progeny, and although the intermediate steps in this process are controversial (Ceredig et al., 2009) the process includes an early segregation of the megakaryocyte and erythroid lineages from the remaining lineages. Commitment to the lymphoid or myeloerythroid lineages results in further expansion of these progenitors during differentiation, and subsequent restriction to specific lineages results in precursor cells with limited proliferative potential.

EMBRYOLOGY OF HEMATOPOIESIS

Blood cells develop in essentially the same manner in embryos of all mammals. Embryonic mammalian hematopoiesis can be divided into three distinct phases: mesoblastic, hepatic, and myeloid (Wintrobe, 1967). The *mesoblastic phase* (also known as the *vitelline phase* because of the predominance of morphologically recognizable hematopoietic cells in the yolk sac) persists for about 10 weeks in human embryos (about 12 days in the mouse). During this time, the predominant blood cell type observed morphologically is the primitive (nucleated) erythrocyte in the yolk sac, which can be detected as early as 18 days of gestation in humans. During the *hepatic period* (beginning at 6 to 8 gestational weeks in the human or 10 to 12 days in the mouse), the fetal liver assumes the major responsibility for blood formation and continues to be hematopoietic until shortly before birth. In humans, a transient period of splenic hematopoiesis precedes the *myeloid phase*, which initiates in marrow cavities at 10 to 12 weeks of gestation (15 to 16 days in the mouse), and by 20 weeks the majority of blood formation in human embryos occurs in the bone marrow. While splenic hematopoiesis is only a transient stage during human development, the spleen remains

hematopoietically active throughout the adult life of the mouse. Despite this, the bone marrow remains the primary site of blood formation, as it contains at least 10-fold higher levels of assayable progenitors compared to the spleen.

As discussed above, the primary anatomical site where hematopoiesis is first observed in mammalian embryos is extra-embryonic, in the numerous blood islands of the yolk sac. The liver primordium is subsequently seeded by migrating HSCs and rapidly becomes the predominant site of embryonic blood production. Recent experimental evidence suggests that a separate origin of hematopoiesis is also present intra-embryonically in mammals, as is the case in lower vertebrates. Intra-embryonic hematopoiesis in mammals is localized in a specialized splanchnopleural region of mesoderm that includes the dorsal aorta and the mesonephros and genital ridge (AGM region, Fig. 3.2; Muller et al., 1994). Although morphologically recognizable hematopoietic elements are present only in the yolk sac during early embryogenesis, progenitor assays can detect hematopoietic activity in the AGM region prior to the appearance of progenitors in the fetal liver (Medvinsky and Dzierzak, 1996). It is currently unclear whether the fetal liver is colonized by HSCs deriving from the yolk sac, the AGM, or both. One series of experiments demonstrated lymphoid and multipotent myeloid generative activity in the region of the dorsal aorta prior to establishment of circulation in the mouse embryo (Cumano et al., 1996). Other studies question the presence of HSC function within the embryo prior to the onset of circulation, when yolk sac-derived cells are rapidly distributed within the embryo (Ghiaur et al., 2008; Lux et al., 2008). However, the biomechanical forces associated with blood flow have also been shown to promote hematopoiesis (Adamo et al., 2009; North et al., 2009), leading to a caveat in the interpretation of studies that rely on interruption of circulation to prevent the distribution of yolk sac-derived blood progenitors (Lux et al., 2008; Rhodes et al., 2008). A recent study utilized inducible labeling in vivo to demonstrate that the yolk sac contributes to the adult HSC pool, without ruling out the AGM as an additional source of HSCs (Samokhvalov et al., 2007). Finally, the endothelial cell origin of HSCs has been demonstrated in a number of studies (Chen et al., 2009; Eilken et al., 2009; Lancrin et al., 2009; Rhodes et al., 2008; Zovein et al., 2008). Therefore, it seems clear that mammalian hematopoiesis initiates within the developing embryo as well as in the yolk sac and placenta. The relative contributions of each of these sites of HSC generation to the adult HSC pool remains to be definitively determined.

Transplantation of yolk sac cells into irradiated adult animals does not result in engraftment. However, HSC activity has been demonstrated in early (day 10) mouse yolk sac after transplantation into neonatal recipient animals, possibly because of an obligate localization of these cells in the hematopoietic liver for further maturation into HSCs prior to marrow colonization (Yoder and Hiatt, 1997). It is likely that the yolk sac promotes primarily primitive hematopoiesis (nucleated red cells that transiently

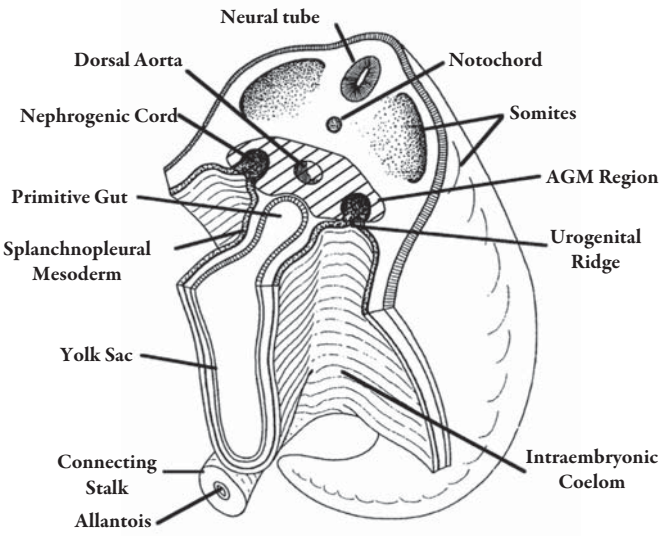

Figure 3.2 Morphology of early mammalian embryogenesis and the origins of the blood-forming cells. The embryo depicted here is human and at the beginning of the fourth week of gestation, just prior to differentiation of the mesonephros and genital ridge from the nephrogenic cord. While morphologically distinct blood cells are already present in the blood islands (not depicted here) of the yolk sac, no blood production is evident in the body of the embryo. Surgical dissection of the AGM region (hatched area in the figure) followed by specific assays for hematopoietic function has demonstrated that primitive hematopoietic stem and progenitor cells are also present in the AGM, even though no morphologically identifiable blood cells can be detected. (Adapted from *Illustrated Human Embryology*, *Volume 1*: *Embryogenesis*, by Tuchmann-Duplessis et al. [translated by L. S. Hurley], 1975, figure 1, page 38. © 1971 by Masson and Company. Used with kind permission of Springer Science and Business Media.)

function during embryogenesis) while the embryonic sources of hematopoiesis are predominantly definitive (the stable, long-term source of hematopoiesis). However, after the onset of blood circulation both types of hematopoietic function are present in both anatomical locations during subsequent development.

The AGM and the yolk sac are both mesodermally derived. The primordial cells that initiate formation of the hematopoietic system migrate to the AGM and yolk sac from the caudal portion of the early primitive streak during gastrulation (Bloom, 1938; Huber et al., 2004). The proliferating cells of the blood islands differentiate along two distinct pathways: one to form the endothelial cell boundaries of the first blood vessels at the periphery of the blood islands, and the other to give rise to primitive blood cells in the center of the islands. Thus, the common mesenchymal ancestry of endothelium and blood cells can be traced to a relatively late period during their ontogeny. Within the mammalian embryo, clusters of hematopoietic cells are not observed in the AGM region but rather are seen intravascularly as clumps of cells attached to the aortic endothelium (Dzierzak and Medvinsky, 1995; Oberlin et al., 2002). In birds, the onset of intra-embryonic hematopoiesis is morphologically obvious, and for many years this was generally felt to be a fundamental difference between mammalian hematopoiesis and that of lower vertebrates.

BONE MARROW AND THE NICHE

Bone is a calcified extracellular matrix of collagen and glycosaminoglycans that is synthesized by osteoblasts—bone-forming cells. The medullary cavity of bone may be hematopoietically active and contain so-called red marrow, or it may be predominantly inactive and filled with fat cells (white marrow). During the process of aging in humans, the anatomical sites of medullary hematopoiesis are progressively limited, beginning at birth, when hematopoietic activity is distributed throughout the skeleton. By 18 years of age, most hematopoietically active marrow is found in central locations such as the pelvis, sternum, and ribs (Amos and Gordon, 1995). The medullary cavity and Haversian canals of bone, which house blood vessels, are lined with a membrane called the *endosteum*. Occasional osteoclasts, which destroy bone, are found in the walls of the medullary cavity and are associated with areas of bone resorption.

The endosteum and its associated osteoblasts are of particular interest because the most primitive HSCs for blood formation appear to be localized near the walls of the medullary cavity (Askenasy et al., 2002). The process of hematopoiesis depends on HSCs in an intimate association with nonhematopoietic tissue cells in the medullary cavity of bone and, in some rodents, in the spleen. These tissue cells are generally termed *stromal cells*, a generic term that may be applied to any of a wide variety of nonmobile cells. The matrix of the medullary cavity includes structural elements of the blood vascular system, nerve fibers, and a system of reticular cells and fibers. This matrix is established during embryogenesis before the initiation of hematopoiesis. Thus, it provides a specialized microenvironment that supports hematopoietic cells within the parenchyma.

The distinct functions of HSCs and the microenvironments that support them are clearly seen in two mutant mouse strains that were selected after mutations at the "dominant spotting" (W) and "steel" (Sl) loci on chromosomes 5 and 10, respectively (Shultz and Sidman, 1987). The products of these two loci interact to result in normal hematopoietic development and function; the Sl gene product is expressed by the microenvironment, while the W gene product is expressed by the hematopoietic cells (Witte, 1990).

The bone marrow microenvironment has several distinct functions with regard to hematopoiesis (Fig. 3.3). First, it must provide conditions to maintain pluripotent HSCs in a primitive state throughout an animal's lifetime, thus ensuring an adequate supply of the seeds of hematopoiesis. Second, it must provide appropriate inductive signals for primitive HSCs to direct regulated development of erythroid, myelomonocytic, and B-lymphoid lineages. The processes of maintenance and differentiation of HSCs must be balanced to sustain a regulated frequency of functionally mature blood cell populations without depleting the HSC pool. The role of regulating the maintenance and differentiation of HSCs is filled by the stromal-cell elements of the marrow microenvironment, although in the case of erythropoiesis the kidney is also intimately involved in an endocrine manner through the production of erythropoietin. In addition to producing cytokines, marrow stromal cells also mediate proliferation and differentiation of hematopoietic progenitors via direct cell–cell interactions, using both common and specialized cell adhesion molecules.

Over the past few years, the characteristics of the bone marrow niche critical for HSC maintenance have been defined experimentally. Two distinct niches in the marrow have been proposed, one associated with the vascular sinuses (Kiel et al., 2005) and the other associated with the

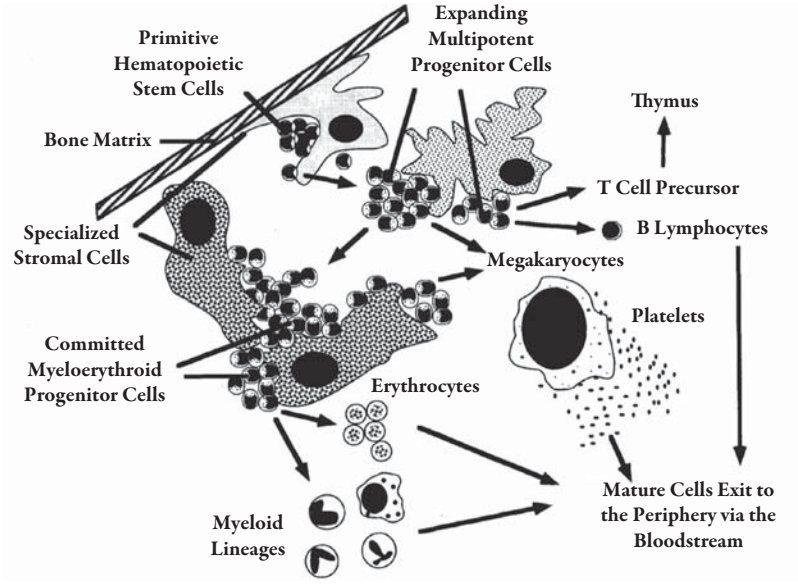

Figure 3.3 The bone marrow microenvironment includes a complex mixture of parenchymal cells that support maintenance and differentiation of hematopoietic cells by secretion of cytokines as well as via cell–cell interactions. The associations between developing hematopoietic cells and their supportive microenvironment are largely uncharacterized; this diagram is strictly a generalized representation of the notion that interactions with stromal cells are thought to regulate various stages of hematopoietic development in specific manners.

endosteum. Osteoblasts have been shown to be an integral part of the endosteal niche (Calvi et al., 2003; Zhang et al., 2003). Moreover, the frequency of HSCs seems to be dependent on the number of osteoblasts (Visnjic et al., 2004; Zhang et al., 2003). Parathyroid hormone was reported to increase hematopoiesis by inducing the synthesis of jagged 1, which serves as a ligand for notch signaling (Calvi et al., 2003). Calcium receptor expression by HSCs facilitates engraftment of transplanted HSCs in response to extracellular calcium concentration (Adams et al., 2006). Conversely, osteopontin serves as a negative regulator, with defective mice showing increased jagged 1 and angiopoietin 1 expression (Stier et al., 2005). Of particular interest to the field of primary immunodeficiencies, the ATM protein, defective in ataxia-telangiectasia, was recently reported to regulate the reconstitutive capacity of HSCs (Ito et al., 2004). Recent insights into the specific hematopoietic microenvironments with the bone marrow have also been provided by vital microscopy studies and by antibodies that detect HSCs and their stromal environments (Calvi et al., 2003; Chan et al., 2009; Kiel et al., 2005; Kohler et al., 2009; Xie et al., 2009; Zhang et al., 2003).

SPLEEN

Another example of the importance of HSC–stromal cell interactions in regulating hematopoietic growth is provided by the mouse spleen. In mice, hematopoietic activity is maintained in the spleen throughout adult life. Although both the bone marrow and spleen support erythropoiesis and granulopoiesis, the spleen is dominantly erythropoietic, whereas granulopoiesis exceeds erythropoiesis in the bone marrow. Implantation of bone fragments into irradiated spleens allows the two types of microenvironments to exist in juxtaposition. In such cases, individual colonies of hematopoietic cells that arise at the border of the two microenvironments are frequently granulocytic in the vicinity of the bone fragment and erythroid in the splenic area (Wolf and Trentin, 1968). Results from these experiments argue that microenvironmental differences can dictate the developmental pathways followed by individual multipotent cells.

Other examples of microenvironments that induce specialized hematopoietic growth include the B- and T-lymphocyte lineages, which develop predominantly in bone marrow and thymus, respectively. These observations are consistent with an "instructive" role for microenvironments in directing lineage commitment (Metcalf, 1998). An equally compelling case can be made for a stochastic model of hematopoietic differentiation (Enver et al., 1998), and it is likely that both mechanisms function in various lineages and stages of development.

HEMATOPOIESIS IN CULTURE

The establishment of long-term cultures of bone marrow stromal elements has provided further evidence that specialized stromal cells contribute to microenvironments that support hematopoiesis. In addition, culture systems have been developed that selectively support HSC maintenance with myeloid and erythroid development (Dexter and Lajtha, 1974), B-cell development (Whitlock and Witte, 1982), or T-cell development (Schmitt and Zuniga-Pflucker, 2002). These culture systems involve the establishment in vitro of specialized stromal cell monolayers or reaggregation of stromal cells and progenitors into a three-dimensional structure to mimic the organ culture of the intact thymus. Stromal cell monolayers may be heterogeneous, consisting of fibroblasts, endothelial cells, adipocytes, and mononuclear-derived macrophages and dendritic cells, or they may be established in vitro as cell lines of clonal origin that have been screened and selected to optimally support hematopoietic development.

Early observations of the myeloid cultures developed by Dexter et al. indicated that adipocytes were most closely associated with hematopoiesis in vitro. The subsequent development of stromal cell lines (Hunt et al., 1987; Kodama et al., 1992) has strengthened these findings in that the stromal cell lines that support in vitro growth of early lymphoid and myeloid cells and maintain spleen colony-forming cells are adipocyte-like and resemble adventitial reticular cells. These latter cells are fibroblastoid cells that form a cellular network, or reticulum, within the bone marrow cavity. They accumulate neutral lipid deposits under certain conditions and are secretory, producing collagen and other proteins (Hunt et al., 1987). A recent study characterized cell populations within developing fetal bone and found that cells co-expressing α_v (an adhesion molecule), CD105 (an endothelial cell marker), and CD90 (a marker useful for identifying HSCs) in the absence of a series of other antigens were capable of establishing hematopoietic niches when implanted under the kidney capsule of adult animals (Chan et al., 2009). This and other model systems promise to provide clues that will clarify the roles of cell–cell interactions and of soluble growth factors in regulating hematopoiesis in general and the development of the immune system in particular.

ISOLATING MOUSE HEMATOPOIETIC STEM CELLS

Because of the important biological and medical implications inherent in the concept of a rare population of primitive cells being responsible for continuous replenishment of all circulating blood cells, efforts to identify and isolate HSCs date back to the first quantitative assays of hematopoietic development (reviewed in Spangrude, 1989). After description of the in vivo spleen colony-forming assay (Till and McCulloch, 1961), development of an in vitro colony assay followed a few years later (Bradley and Metcalf, 1966). Although early efforts to enrich primitive hematopoietic cells relied on these assays, the limitations of the assays in reflecting the self-renewal potential of HSC were recognized early on. Velocity and equilibrium centrifugation studies demonstrated that bone marrow cells with an inherent capacity to produce splenic colonies could be separated from those capable of in vitro colony formation (Worton et al., 1969a, 1969b) and that self-renewal potential, as indicated by the content of splenic colony-forming units

(CFU-S) within individual spleen colonies, could be separated from the bulk of the CFU-S in normal bone marrow (Worton et al., 1969a). These observations indicated that CFU-S activity might not directly correlate with self-renewal potential, a problem that has not been conclusively resolved. However, the early separation studies demonstrated that cell-separation methods could indeed be applied to dissect the hierarchy of primitive hematopoietic cells.

Mouse HSCs can be enriched from adult bone marrow by use of a number of methods. In one approach to the identification and enrichment of mouse HSCs, monoclonal antibodies specific for antigens that characterize cells belonging to specific mature and committed hematolymphoid lineages are used to identify these cells in bone marrow suspensions (Müller-Sieburg et al., 1986). The marked cells can be depleted by a solid-phase immunological method such as panning (Jordan and Lemischka, 1990) or by the use of immunomagnetic particles (Bertoncello et al., 1989; Ikuta et al., 1990). The remaining population of cells, termed *lineage-negative* (Lin^{neg}), contains approximately 5 percent of the initial number of bone marrow cells and consists of a mixed population of multipotent HSCs, early progenitors, and late progenitors. Lin^{neg} cells can be further fractionated with specific monoclonal antibodies that recognize antigens expressed by a variety of hematopoietic and nonhematopoietic cells—for example, Thy-1 (Müller-Sieburg et al., 1986), Sca-1 or Ly-6A/E (Spangrude and Brooks, 1992), c-kit (Okada et al., 1991), and major histocompatibility complex class I molecules (Mulder et al., 1984). The lectin wheat germ agglutinin has also been useful in discriminating multipotent HSCs from early- and late-stage progenitors due to a relatively high number of binding sites for the lectin on multipotent HSCs compared to that on later-stage progenitors (Visser et al., 1984). When combined with a depletion of cells expressing high levels of lineage differentiation antigens, any one of these markers alone, or in combination, will identify a group of primitive hematopoietic cells in adult bone marrow or fetal liver. This multiparameter approach is helpful because few cell-surface antigens are unique to HSCs (Kiel et al., 2005), and most currently defined HSC markers continue to be expressed as the cells differentiate and new, lineage-specific markers appear.

HETEROGENEITY OF THE HEMATOPOIETIC STEM CELL COMPARTMENT

Cell populations resulting from antibody selection techniques are heterogeneous in function, suggesting a complex organizational structure within the HSC compartment (Fig. 3.1). The heterogeneity is mostly with respect to self-renewal potential, since a high frequency of cells isolated by antibody enrichment are multipotent for both lymphoid and myeloid lineages (Spangrude and Johnson, 1990). The subset of multipotent cells possessing extensive self-renewal potential is resistant to killing by 5-fluorouracil and other cell-cycle–active agents (Hodgson and Bradley, 1979). This finding suggests that these cells are metabolically quiescent or that they possess elevated levels of a multidrug-resistance mechanism (Chaudhary and Roninson, 1991).

Several methods have been used to select for long-term repopulating HSCs from antibody-enriched stem/progenitor-cell populations. The vital mitochondrial dye rhodamine-123 can be used to distinguish cell populations on the basis of metabolic activity, and self-renewing HSCs are predominantly recovered from the group of cells exhibiting low mitochondrial staining (Bertoncello et al., 1985). Rhodamine-123 is also a substrate for multidrug efflux pumps. However, it is likely that the discrimination between the self-renewing and non–self-renewing HSC subsets by rhodamine-123 is due to differences in mitochondrial content and activity (Kim et al., 1998). In a second strategy, vital nucleic acid dyes such as Hoescht 33342 are used to identify quiescent cells based on cell-cycle position (Neben et al., 1991; Wolf et al., 1993) or dye efflux that defines a "side population" of cells by flow cytometry (Goodell et al., 1997). Finally, specific combinations of antibody markers can select for self-renewing HSCs (Morrison and Weissman, 1994). The combination of cell-surface and metabolic markers allows the recovery of a population of cells capable of long-term repopulation of irradiated animals after transplantation of less than 10 cells (Ema et al., 2005; Spangrude et al., 1995; Wolf et al., 1993). In contrast, over 10^5 normal bone marrow cells are required to produce a similar level of reconstitution.

The published methods for enriching populations of HSCs from mouse bone marrow and fetal tissues have dramatically improved the ability of researchers to explore the early events in normal mammalian hematopoiesis. As more enriched populations have become available for experimental use, it has become possible to critically test these cells for their ability to respond to recombinant cytokines by proliferating (Li and Johnson, 1992; Sitnicka et al., 1996), to assess the developmental pathways that the cells will follow under defined conditions in vitro (Yamazaki and Nakauchi, 2009), and to explore the ability of purified cells to home to marrow niches in vivo (Adams et al., 2009).

CHARACTERISTICS OF HUMAN HEMATOPOIETIC STEM CELLS

The demonstration that the CD34 antigen is expressed by progenitor cells in human bone marrow (Civin et al., 1984) has allowed many investigators to explore the HSC compartment in humans. Applying the same general approach that was successful in the mouse, several groups have combined negative selection for lineage markers; positive selection for CD34 (Andrews et al., 1986), Thy-1 (Baum et al., 1992), or c-kit (Briddell et al., 1992); and selection for low staining with rhodamine-123 (Srour et al., 1991) to achieve high enrichments of human hematopoietic progenitor cells. Positive selection for CD34 alone has been used as a method to enrich for progenitors prior to bone marrow transplantation after chemotherapy for a number of malignancies (Berenson et al., 1991; Shpall et al., 1994).

Highly purified $CD34^+$ $Thy\text{-}1^+$ Lin^{neg} cells have also been used clinically for transplants after myeloablative chemotherapy (Michallet et al., 2000). In allograft transplants for treatment of immunodeficiencies, selection for HSCs by specific antigen expression may decrease the potential for graft-versus-host disease (Flake et al., 1996). In addition, the introduction of normal counterparts of defective endogenous genes into the hematopoietic system for treatment of immunodeficiencies (see Chapters 60 and 61 of this volume) requires that the target population be relatively enriched for self-renewing HSCs.

In recent years, a great deal of attention has been focused on augmentation and acceleration of engraftment in bone marrow transplant settings by cytokine therapy in the recovery phase, and on the use of peripheral blood-derived stem/progenitor cells as a source of hematopoietic stem cells for human transplantation. While hematopoietic progenitors are rarely found circulating in peripheral blood under normal circumstances (Micklem et al., 1975), cytotoxic drug treatment, the administration of hematopoietic cytokines (Bodine et al., 1993), or both treatments in sequence (Siena et al., 1989) induce a rapid peripheralization of hematopoietic stem/progenitor cells. These cells can be harvested by multiple leukopheresis sessions over several days, frozen for storage, and infused to mediate hematopoietic recovery following marrow ablative therapy. There is a significant effect of peripheral blood-derived stem and progenitor cells on the kinetics of recovery during the pancytopenic phase, when infection and hemorrhage can result in significant patient morbidity and mortality. A variety of studies have documented dramatic effects on both neutrophil and platelet recovery when peripheral blood stem cells, with or without supplemental cytokines, were used to mediate hematopoietic recovery following high-dose chemotherapy (Basser et al., 1996; Benjamin et al., 1995). Enrichment of HSCs from normal donor peripheralized blood progenitor products (Murray et al., 1995; Weaver et al., 1995) may enable efficient allotransplantation without graft-versus-host disease in the treatment of many immunodeficiencies (Flake et al., 1996).

ASSAYS FOR HEMATOPOIETIC STEM CELLS

A great deal of attention has been focused on the development of assay systems that specifically detect the activity of the most primitive of HSCs for hematopoiesis (Fig. 3.4). One approach to an in vivo clonal assay for long-term repopulation is limiting dilution competitive repopulation to derive a measure of competitive repopulating units (CRUs) (Szilvassy et al., 1990). In this assay, very small numbers of genetically marked cells are transferred into anemic W/Wv mice (Boggs et al., 1982) or irradiated animals. In the latter case, a radioprotective dose of normal marrow cells is also provided to mediate radioprotection, and the development of donor-derived populations is followed over long periods of time. This approach has been used to estimate the frequency of long-term repopulating cells in normal marrow suspensions (Boggs et al., 1982; Micklem et al., 1987). Enrichment of mouse HSCs from bone marrow resulted in repopulation of recipient animals after transfer of single injected cells (Osawa et al., 1996; Smith et al., 1991; Spangrude and Weissman, 1988).

Because it is very difficult to determine what the seeding efficiency of this assay is—that is, how frequently an intravenously injected cell seeds to a microenvironment such as the spleen or bone marrow where hematopoiesis is supported—it

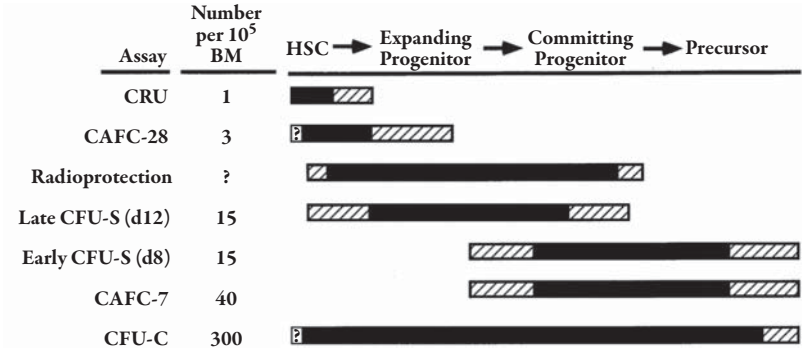

Figure 3.4 Assays that detect various members of the hematopoietic hierarchy. A number of assays currently used to detect hematopoietic stem and progenitor cells are listed along with the approximate frequency of the normal mouse bone marrow cells detected by these assays and a general indication of the level of the hematopoietic hierarchy detected by each assay. The black and shaped portions of the bars indicate strong and weak activity, respectively, in the indicated assay. The assays are abbreviated as follows: CRU, competitive repopulating unit, a transplantation assay that measures the clonal frequency of cells able to contribute long term to blood formation in a transplant recipient. CAFC, cobblestone-area-forming cell, a cell culture assay that measures the ability of stem and progenitor cells to form within 7 to 28 days a morphologically distinct colony of cells in association with a monolayer of cultured stromal cells; Radioprotection, the potential of transplanted stem and progenitor cells to mediate hematological rescue of lethally irradiated animals; CFU-S, colony-forming units (spleen), a transplant assay that measures the formation of clonally derived colonies of proliferating hematopoietic cells in the spleens of irradiated animals 8 or 12 days after transplant; CFU-C, colony-forming units (culture), a culture assay that measures the expansion of clones of cells in a semisolid culture medium in response to soluble growth-factor stimulation. This assay can also be performed by seeding single cells into culture wells. The frequency of responding cells in each assay has been taken from Neben et al. (1993) and Szilvassy et al. (1996); these numbers will vary depending on specific assay conditions and the age and strain of the donor mouse from which bone marrow cells are obtained.

is not possible to know how frequent the long-term repopulating cell was in the original population; only a minimum frequency can be determined based on the measured seeding efficiency of about 35 percent (Camargo et al., 2006). Further, since there is ample evidence that long-term repopulation is usually quasi-clonal in radiation-reconstitution models, the absence of progeny in the long term may indicate that the single injected HSC is one of many in a quiescent state, rather than supporting the idea that the cell was unable to self-renew and give rise to progeny over a long period of time (Van Zant et al., 1992; Wilson et al., 2008). The quasi-clonal repopulation problem compounds the difficulty of measuring the frequency of long-term repopulating cells in transplant models, even when a remarkable level of enrichment for the activity has been achieved.

Very soon after the description of the CFU-S assay by Till and McCulloch in 1961, the question of whether splenic colonies truly represent the proliferation of primitive HSCs became an important issue. Since the criteria for defining HSCs at that time included multilineage potential and a capacity for self-renewal, it naturally followed that one should be able to demonstrate the presence of new colony-forming cells within spleen colonies. This hypothesis was tested in a double transplant experiment by either excising individual spleen colonies (Siminovitch et al., 1963) or pooling the entire spleen from a CFU-S experiment (Siminovitch et al., 1964) for transplant into secondary irradiated recipients. The results revealed a broad heterogeneity in the ability of splenic colonies to initiate new splenic colonies. Also, when a longer period of time was allowed to elapse prior to harvesting spleen colonies for secondary transplant, the number of colonies containing secondary CFU-S increased. Many more colonies harvested 14 days after injection contained CFU-S compared to colonies harvested after 8 days. While a reasonable interpretation at the time invoked an extended timeframe necessary for the self-renewal process within spleen colonies (Lewis and Trobaugh, 1964), later work revealed that the majority of early (day 8) CFU-S disappeared a few days later and that at least half of the late (days 11 to 13) colonies arose from a separate group of hematopoietic cells (Magli et al., 1982; Wolf and Priestley, 1986). These intrinsic differences in CFU-S hinted at the limited utility of the CFU-S assay to detect HSCs; only after a secondary transplant could one be assured of the self-renewing potential inherent in the original spleen colony.

Refinements in HSC characterization and enrichment have strengthened the concept that many types of hematopoietic cells that form spleen colonies lack the ability to reconstitute long-term hematopoiesis in irradiated animals (Ploemacher and Brons, 1988). Conversely, several groups have demonstrated long-term repopulating potential in the apparent absence of splenic colony-forming potential (Jones et al., 1990; Ploemacher and Brons, 1989; Wolf et al., 1993). While it is not yet clear whether inhibitory cytokines (Graham et al., 1990) or splenic seeding considerations (Spangrude and Johnson, 1990) may explain the failure of certain HSC preparations to form spleen colonies, it is very clear that spleen colony formation is not a unique characteristic of primitive HSCs (Hodgson and Bradley, 1979; Magli et al., 1982). Further, the inability to use such an assay in the investigation of human hematopoiesis, with the possible exception of chimeric human-mouse model systems (McCune et al., 1988), dictates the need to develop specific in vitro assays for HSCs.

Many recent attempts to develop specific in vitro assays for HSCs have relied on co-culture of candidate HSC populations with feeder layers of bone marrow-derived stromal cells. A number of laboratories have used this approach to analyze hematopoiesis with mouse and human models (Ploemacher et al., 1989; Sutherland et al., 1990). In this assay there are two phases: an expansion phase, in which the primitive HSC number is increased without a large degree of differentiation, and a detection phase, when the products of the initial expansion are read out. In the expansion phase, self-renewal must be favored over differentiation, and the typical approach is to use a stromal cell feeder layer under the conditions originally defined by Dexter et al. (Dexter and Lajtha, 1974) to culture HSC populations. In most cases, the second phase of the assay involves harvesting the cells that differentiate in the cultures and testing these progeny cells for the ability to produce macroscopic colonies in semisolid medium under the influence of a variety of cytokines (the culture colony-forming unit, or CFU-C assay). While the readout of colony formation is not an activity uniquely associated with HSCs, the differentiation of HSCs in these stromal cell co-cultures is thought to result in the production and thus a net increase in the number of colony-forming cells. The results of such an assay are fairly quantitative, but the assay is not clonal because individual colonies in the second phase of the assay do not reflect single-input HSCs. However, if the co-culture is initiated under limiting dilution conditions, an estimate of the frequency of the HSCs in any given cell population is possible. Furthermore, a higher absolute number of colony-forming cells produced in any one culture is interpreted as an indicator of a more primitive initiating HSC. This culture system, and variants thereof, is usually termed the *long-term culture initiating cell* (LT-CIC) assay.

Co-cultures of HSC and supportive bone marrow stroma evolve to generate unique associations between the two cell types. The mobile HSC interdigitates between and beneath the stromal monolayer, resulting in HSCs with a characteristic nonrefractile appearance by phase-contrast microscopy. These stromal-covered cells proliferate to form clusters of tightly packed cells that have been referred to as *cobblestone areas*, and the assay to detect such events is known as the *cobblestone-area-forming cell* (CAFC) assay (Neben et al., 1993; Ploemacher et al., 1989). The frequency of formation of cobblestone areas can be correlated to hematopoietic repopulating activity in vivo, and the kinetics of cobblestone area formation reflects the relative maturation stage of the initiating cells, with more primitive cells requiring a longer period of time to establish a cobblestone area. These observations have been incorporated into the CAFC assay, which does not rely on a readout of CFU-C, but rather quantitates the frequency of cobblestone areas as a function of time (Neben et al., 1993; Ploemacher et al., 1991; Weilbaecher et al., 1991). There is currently no strong evidence that stromal cell systems effectively reproduce the bone marrow environment in terms of self-renewal of HSCs (Spooncer et al., 1985). The stromal co-cultures can

produce CFU-C from input HSCs over prolonged periods of time (van der Sluijs et al., 1990), but it is unclear to what extent HSCs can actually self-renew in these cultures relative to the in vivo environment.

While some correlation has been made between long-term reconstitution of lethally irradiated animals and some in vitro culture systems (Ploemacher et al., 1991), in fact the only definitive method available to define the self-renewing characteristics of HSCs is by transplantation in vivo (Orlic and Bodine, 1994). The availability of genetically defined strains of mice that allow easy identification of donor-derived cells in the peripheral blood of recipient animals in an otherwise syngeneic transplant (Harrison, 1980, Spangrude et al., 1988) provides a valuable model system for HSC function that has not yet been entirely duplicated by in vitro culture systems (van der Sluijs et al., 1993). The major limitation of the in vitro systems is that, in general, the development of only one or a few hematopoietic lineages is supported. Also, sole reliance on long-term culture initiation as an indicator of HSC function ignores the known capacity of mammalian cells to adapt to tissue culture conditions. A variety of immunodeficient strains of mice have been widely used as an in vivo model for engraftment of both normal and leukemic hematopoietic cells from human sources (Pearson et al., 2008). This assay allows detailed study of stem cell homing to bone marrow and multilineage differentiation in the context of a normal microenvironment.

SELF-RENEWAL OF HEMATOPOIETIC STEM CELLS

A major advantage of using a mouse model system to define basic characteristics of the HSC is that this model can be used to demonstrate the most critical of HSC functions, self-renewal. The concept of self-renewal in hematopoiesis can be interpreted in several ways. One possibility is that self-renewal of HSCs occurs at each cell division, which requires that HSCs divide in the complete absence of differentiation. This leads to the conclusion that one HSC may contribute indefinitely to hematopoiesis, a critical assumption for the application of gene therapy protocols in immunodeficiencies. If, however, one envisions a heterogeneous compartment of HSCs, all of which share the ability to initiate development in multiple hematopoietic lineages but differ in ability to give rise to more multipotent cells, the conclusion is consistent with the clonal succession model as proposed by Kay (1965), and there is less optimism for prolonged correction of genetic defects by gene therapy. An intermediate situation consisting of essential elements from both extremes produces a further variation. It will be difficult to prove or disprove the proposal that one HSC can divide to produce progeny of identical proliferative and developmental potential; however, many experiments have demonstrated the reality of clonal succession and of the heterogeneous nature of the HSC compartment (McKenzie et al., 2006). Sequential activation of HSC clones leads to clonal or quasi-clonal contributions to hematopoiesis, as demonstrated by transplantation experiments between animals differing at isoenzyme loci (Abkowitz et al., 1995; Micklem et al., 1987) or by transplants of bone marrow cells carrying unique retrovirally induced genetic markers (Jordan and Lemischka, 1990). Serial transplantation of bone marrow, which eventually leads to a loss of repopulating activity (Harrison et al., 1990), can demonstrate two distinct phases of engraftment in recipient animals. The first phase is unsustained, apparently because more committed members of the HSC compartment are unable to maintain hematopoiesis in the long term. The second phase is sustained and is due to very primitive HSCs (Jones et al., 1989). These observations are compatible with clonal succession and with the generation-age hypothesis (Rosendaal et al., 1976), which extends the clonal succession model to predict that the number of generations an HSC is removed from its initial progenitor is inversely proportional to its proliferation potential (and hence hematopoietic-repopulating potential) and directly proportional to its state of activation. This means that the ability of any individual HSC to self-renew is limited, but the compartment of multipotent cells possesses the self-renewing ability of the sum of all individual HSCs.

Application of transplantation pressure in hematopoiesis results in exhaustion of HSCs (Harrison et al., 1990). In a very intriguing study, allophenic chimeras, made by aggregating embryos of two inbred mouse strains, were used to show that HSC exhaustion can be observed under normal developmental pressure (Van Zant et al., 1990). In these experiments, one partner mouse strain (DBA/2) has spleen colony-forming cells, of which 24 percent are normally in cell cycle, whereas in the other partner strain (C57BL/6) only 2.6 percent of these cells are in cycle. In allophenic chimeras between these two strains, the DBA/2 HSC population predominated early in life, only to be overtaken and eventually eclipsed by the C57BL/6 population. A similar observation was made after bone marrow transplants from chimeras into irradiated F1 recipients. These experiments bear out basic predictions of the generation-age hypothesis and point to intrinsic differences in the HSC as a factor in the longevity of hematopoiesis and of lifespan. Do HSCs also self-renew at a cellular level? This question is critical to the concept of gene therapy as a permanent cure for immunodeficiencies. To answer this question definitively we will need more sophisticated techniques of cell culture and analysis.

CONCLUSIONS

The outcome of human bone marrow transplantation may be improved if enriched populations of early progenitors and HSCs are transplanted rather than whole bone marrow or apheresis products. This could be true in allogeneic transplants, where graft-versus-host disease might be eliminated by T-cell depletion, and also in autotransplants, where residual tumor cells in the graft may contribute to relapse (Gazitt et al., 1995). Application of gene therapy to human immunodeficiency treatments via transplantation will also require HSC enrichment to improve the efficiency of targeting functional genes to the hematopoietic generative compartment. It is critical that we understand the biology of hematopoiesis and know how to maximize the self-renewing potential of transplanted HSCs.

Researchers working in the human system must currently rely on assay systems for long-term repopulation that have not been thoroughly examined for their specific mechanism of detecting the critical (self-renewing) populations of cells. Unraveling of the mysteries of the HSC compartment has been complicated by the difficulty in obtaining native HSCs in any large quantity for classical cell biological studies. This problem is compounded by the observation that HSC biology (phenotype and function) varies among mouse strains, making concrete rules for early hematopoiesis difficult to formulate.

Several recent studies suggest that bone marrow-derived hematopoietic stem cells may have broader developmental potential than originally thought, as examples of differentiation into epithelium, hepatocytes, neurons, and muscle have been reported (Krause et al., 2001; Lagasse et al., 2001). The phenomenon of differentiation across lineage barriers is often referred to as *plasticity* or *transdifferentiation*. Furthermore, evidence suggests that multipotent cells capable of generating blood and other tissues exist in many adult tissues (Anderson et al., 2001). Although these new observations may open new avenues to treatment of genetic diseases, this field is still highly controversial, with many reports describing both the existence and the essential lack of plasticity. In some cases fusions between cells have been found as the underlying mechanism (Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003). The ability to reprogram fully differentiated mammalian cells to a pluripotent state similar to embryonic stem cells (Yamanaka, 2009) demonstrates that lineage plasticity is in fact a reality that may help open more opportunities for cellular therapy of primary immunodeficiency diseases in the future.

The marked propensity of HSCs to rapidly differentiate in most in vitro culture systems (Rebel et al., 1994) has hampered our ability to investigate under controlled, in vitro conditions the hierarchy of the HSC compartment and to approach the question of how to maintain the essential "stemness" of HSC populations. The possibility that self-renewal in the HSC compartment is limited to a finite number of cell divisions may indicate that even under the best conditions unlimited expansion of true HSCs is an impossible goal to attain. On the other hand, recent studies have begun to reveal transcriptional mechanisms that might be exploited to better regulate HSC self-renewal (Essers et al., 2009; Liu et al., 2009; Luis et al., 2009; Milsom et al., 2009). This possibility has an obvious impact on proposals for gene therapy of immunodeficiencies through transplantation, since self-renewal of transduced stem cells is the only vehicle by which this may be accomplished. Development of better clinical methods for management of the complications of allogeneic transplants will also be an important future direction for effective treatment of congenital immunodeficiencies.

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4.

T-CELL DEVELOPMENT

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INTRODUCTION

The purpose of this chapter is to give a broad overview of the major stages of T-cell development. T-cells are key regulators of acquired immunity, and their functions are determined by events that occur during an intricate developmental process in the thymus (Petrie and Zuniga-Pflucker, 2007). An understanding of T-cell development is fundamental to our understanding of immune responses. The interaction of the T-cell receptor (TCR) expressed by a developing T-cell (thymocyte) with self-antigens presented by stromal cells in the thymus determines the ultimate fate of that thymocyte (Starr et al., 2003). Only a small fraction of thymocytes become the mature T-cells that patrol the body's periphery and respond to foreign antigen. The molecular interactions that lead to the elimination of self-reactive thymocytes ("negative selection") and the survival of those that can potentially mount protective immune responses ("positive selection") are complex (Starr et al., 2003).

Our understanding of T-cell development has been greatly enhanced through the use of genetic tools, particularly mutant mouse models such as transgenic mice and gene-targeted or "knockout" mice (Mak et al., 2001). Additionally, T-cell development has been further dissected with the help of in vitro culture systems such as fetal thymic organ culture (FTOC) (Hare et al., 1999) and the OP9-DL1 cell system (Zuniga-Pflucker, 2004). We start this chapter with overviews of the TCR proteins and the genes encoding them, followed by a brief discussion of the co-receptors CD4 and CD8, which have enormous influence on T-cell development. Finally, we describe the stages of T-cell development in detail at both the cellular and molecular levels. This chapter deals with mouse models of T-cell development; thus, the reader is referred to recent reviews on human T-cell development (Sitnicka, 2009; Spits, 2002; Taghon and Rothenberg, 2008) for further information on this topic.

TCR/CD3 COMPLEX

The TCR is responsible for the recognition of both foreign and self-antigens and is central to both T-cell development and the mounting of specific immune responses. Inability to recognize foreign antigen can lead to overwhelming infection or cancer, while inappropriate recognition of self-antigens can result in autoimmune disease. The binding of antigen, in the form of peptide presented by a major histocompatibility molecule (MHC), by a TCR triggers a complex intracellular signaling cascade that leads to the activation of transcription factors in the T-cell's nucleus (Acuto and Cantrell, 2000). These transcription factors drive gene expression programs that govern the fate and functions of these T-cells, whether they be immature thymocytes developing in the thymus (Rothenberg, 2007) or mature T-cells mediating immune responses in the body's periphery (Smith-Garvin et al., 2009).

TCRs are heterodimeric proteins composed of either TCRα and β chains, or TCRγ and δ chains. In mice, thymocytes and the majority of peripheral T-cells bear $\alpha\beta$ TCRs, but about 5 to 10 percent of all T-cells bear $\gamma\delta$ TCRs. With a few exceptions, TCR$\alpha\beta$ heterodimers recognize peptides complexed with MHC molecules, but some TCR$\gamma\delta$ molecules recognize nonpeptide antigens either directly or bound to the MHC-like molecules (Chien and Konigshofer, 2007). For both $\alpha\beta$ and $\gamma\delta$ TCRs, recognition of the antigen depends on the binding site formed by portions of the $\alpha\beta$ or $\gamma\delta$ chains (Hayday and Tigelaar, 2003). However, surface expression of the TCR and antigen-specific T-cell signaling and activation depend on the association of the TCR with the CD3 complex. The CD3 complex consists of at least five distinct membrane

proteins, CD3γ, δ, ε, ζ, and η, which are noncovalently associated both with each other and the TCR heterodimer (Kuhns et al., 2006). The CD3 γ, δ, and ε chains are found as γε and δε subunits within the TCR/CD3 complex, while the ζ chain is found as a homodimer (in 90 percent of TCRs) or as a ζ heterodimer (~10 percent of TCRs) (Malissen and Malissen, 1996). The CD3ζ and η chains are splice variants derived from the same gene. The minimal stoichiometry of the most common αβ TCRs is TCRαβ: CD3γεCD3δεCD3ζ (Fig. 4.1). All CD3 chains contain at least one of the tyrosine-rich ITAMs (immunoreceptor tyrosine-based activation motifs) essential for initiating intracellular signaling following antigen binding to the TCR (Kuhns et al., 2006; Smith-Garvin et al., 2009).

TCR GENES

An αβ TCR binds to peptide-MHC (pMHC) via its variable (V) region located at the extracellular N-terminus of the heterodimer. The V region is linked to an invariable extracellular region, a transmembrane domain, and a short intracellular domain at the carboxyl terminus of the protein. These latter elements are encoded by the constant (C) region exons of the TCR loci. The V regions of both component TCR chains contribute to the antigen binding site, and each chain's V region is encoded by a V exon. Analogous to the immunoglobulin (Ig) light chain gene, the TCRα and γ V exons contain a V (variable) and a J (joining) gene segment, while, like the Ig heavy chain gene, the TCRβ and δ V exons contain a V, a D (diversity), and a J gene segment. In each developing thymocyte, the V and J or V, D, and J gene segments forming the V exons are randomly chosen from the huge array of multiple V, D, and J gene segments present in germline TCR loci. In a process known as "somatic gene recombination," the RAG1/2 genes are key players that initiate the process of gene recombination (Chen et al., 1994; Schatz et al., 1989). It is the random rearrangement of V(D)J gene segments that generates most of the enormous diversity of TCRs expressed on mature T-cells. Additional diversity is introduced at V-D-J junctions through the introduction of template-independent (or N) nucleotides during V(D)J rearrangement. N-nucleotide addition is mediated by the enzyme terminal deoxynucleotidyl transferase (TdT) (Tonegawa, 1983). The stage-specific timing of when these TCR DNA recombination events take place during T-cell development will be discussed later.

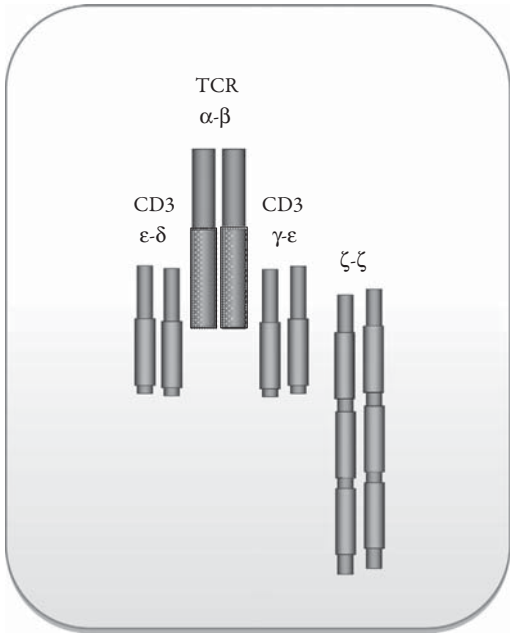

Figure 4.1 αβ TCR/CD3 complex on the surface of T-cells. Schematic drawing of the TCR/CD3 complex in the T-cell membrane. The TCR heterodimer is typically associated with three other CD3 dimers: εδ, γε, and ζζ. The immunoreceptor tyrosine-based activation motif (ITAM) regions are depicted within the CD3 chains. (See Color Plate.)

THE CO-RECEPTORS

Although the TCR antigen binding site is responsible for the recognition of specific peptide in the binding groove of an MHC molecule, the CD8 and CD4 molecules function as germline-encoded cognate receptors that bind to nonpolymorphic regions of MHC class I and MHC class II molecules, respectively (Germain and Stefanova, 1999). The co-receptors facilitate TCR signal transduction because the cytoplasmic domains of both CD4 and CD8 are physically associated (with differing stoichiometry) with Lck tyrosine kinase (Veillette et al., 1988; Weiss and Littman, 1994). Both CD4 and CD8 have important roles in the ontogeny and selection of thymocytes and in the activation of mature T-cells resident in peripheral lymphatic organs (Germain and Stefanova, 1999; Wallace et al., 1993).

CD8 and MHC Class I

CD8 is a cell surface glycoprotein expressed on MHC class I-restricted T-cells. In mice, thymocytes and peripheral T-cells generally express CD8 as heterodimers of CD8α and CD8β. The MHC class I molecule to which CD8 binds is a heterodimer composed of an MHC class I α chain noncovalently associated with the invariant β2-microglobulin (β2M) chain. β2M is crucial for the expression of the MHC class I molecule on the cell surface. The peptides associated with MHC class I molecules are generally derived from endogenous proteins degraded by proteasomes in the cytosol. The peptides are translocated into the endoplasmic reticulum (ER) by a heterodimeric transmembrane transporter complex with structural similarity to ATP binding cassette transporters (Spies and DeMars, 1991). The TAP1 (transporter associated with antigen processing-1) and TAP2 subunits of this transporter complex are encoded by the *Tap1* and *Tap2* genes, which map to the MHC class II region (Deverson et al., 1990; Monaco et al., 1990). Once in the ER lumen, the endogenous peptides are immediately loaded into the binding grooves of newly synthesized MHC class I molecules (Androlewicz and Cresswell, 1996).

Because interaction with MHC class I is required for $CD8^+$ T-cell development, disruptions in either the β2M gene or the *Tap* genes have severe effects on the mature $CD8^+$ T-cell

population. For example, a drastic reduction in the number of $CD8^+$ T-cells is observed in gene-targeted β2M-deficient mice (Koller et al., 1990; Zijlstra et al., 1990), and a similar phenotype is observed in animals in which the *Tap1* gene has been deleted (Van Kaer et al., 1992). Cytotoxic T-cell functions, which are mainly carried out by $CD8^+$ T-cells, are severely impaired in these mutant mouse strains because these cells fail to be generated in the absence of CD8–MHC class I interactions; however, T-helper activities mediated by $CD4^+$ lymphocytes remain normal.

CD4 and MHC Class II

Murine CD4 is a 55 kDa single-chain glycoprotein with four Ig-like extracellular domains, a transmembrane region, and a cytoplasmic domain (Littman, 1987). CD4 molecules interact with MHC class II molecules in both functional studies and binding assays. The two most distal extracellular domains of CD4 bind to the nonpolymorphic β2 domain of MHC class II molecules (Konig et al., 1996). The intracellular domain of CD4 is associated with large quantities of Lck, making it particularly important for the initiation of the complex TCR signaling cascade (Salmond et al., 2009). The majority of T-helper functions, which consist primarily of the secretion of cytokines required for the complete activation of B-cells and cytotoxic T-cells, are carried out by $CD4^+$ T-cells.

The MHC class II molecule to which CD4 binds is a heterodimer composed of an MHC class II α chain noncovalently associated with an MHC class II β chain. MHC class II molecules generally present peptides from exogenous sources. Since both MHC class I and II molecules are assembled in the ER, and both are capable of binding peptides, a mechanism must exist in the ER to protect the peptide-binding groove of class II molecules from occupancy with endogenous peptides (Neefjes and Ploegh, 1992). The invariant chain (Ii) is coordinately expressed with MHC class II in the ER and associates with newly synthesized MHC class II molecules such that the binding of peptide in the MHC class II groove is blocked. As the MHC class II–Ii complexes traverse the increasingly acidic intracellular antigen-processing compartments, Ii undergoes stepwise degradation by cathepsin-like proteases until only a fragment of Ii called CLIP (class II-associated invariant chain peptide) remains in the binding groove (Roche, 1995). CLIP is removed from the binding grove by the action of H2-M or HLA-DM in humans. Both H2-M and HLA-DM are heterodimers with low homology to conventional MHC class II proteins (Roche, 1995). H-2M co-localizes with MHC class II molecules in an endolysosomal compartment called the "MHC class II compartment" (MIIC) (Roche, 1995) and functions as a peptide exchanger, catalyzing the release of CLIP peptides from the MHC class II groove and the subsequent loading of processed exogenous peptides (Denzin and Cresswell, 1995; Sherman et al., 1995). Once the MHC class II molecule is loaded with peptide, H-2M is released and the pMHC complex makes its way to the surface.

The functions of many molecules involved in the exogenous antigen-processing pathway have been clarified through studies of knockout mice. The development and function of $CD4^+$ T-cells is disturbed in mice lacking Ii, confirming the crucial role of this protein in normal MHC class II antigen presentation (Viville et al., 1993). In H2-M-deficient mice, normal amounts of MHC class II molecules are present on the cell surface but the majority of them are associated with CLIP rather than exogenous peptide (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). Unexpectedly, positive selection of MHC class II-restricted $CD4^+$ T-cells appeared intact in these animals and they possessed large numbers of $CD4^+$ T-cells that responded vigorously to antigens presented by normal antigen-presenting cells (APCs). However, APCs from H2-M-deficient mice were unable to stimulate T lymphocytes to respond to any antigen (Fung-Leung et al., 1996). In MHC class II-deficient mice (Cosgrove et al., 1991; Grusby et al., 1991; Kontgen et al., 1993), thymocyte development is blocked at an early stage and $CD4^+$ lymphocytes are virtually absent in peripheral lymphoid organs. However, the development and functions of peripheral $CD8^+$ cytotoxic T-cells are normal. Surprisingly, there were significant numbers of $CD4^+$ T-cells in the thymi of MHC class II-deficient mice, although these cells were not of a mature phenotype. A subset of these unusual $CD4^+$ cells was found to be restricted to MHC class I-like CD1 molecules (Cardell et al., 1995). These cells have been further characterized as natural killer (NK)-T-cells, and they belong to a specialized class of T-cells that uses an invariant TCR to select on CD1 molecules expressed by immature thymocytes (Matsuda et al., 2008). These NKT-cells have a unique cytokine expression profile and may play important functions in tumor recognition and inflammation (Bendelac et al., 2007).

Recently, a mouse model in which both class I and class II MHC molecules along with CD4 and CD8 are deleted was generated (Van Laethem et al., 2007). This quadruple-deficient mouse line showed an interesting outcome of T-cell development, in that mature αβ-T-cells were generated in the thymus, which given the MHC deficiency were selected by and restricted to non-MHC ligands. These findings are discussed below, but they highlight the role of CD4 and CD8 in enforcing cognate MHC restriction by the TCR (Singer et al., 2008).

OVERVIEW OF T-CELL DEVELOPMENT

In this section, we present a brief overview of the major stages of T-cell development, in order to better orient the reader for the more detailed discussions of thymocyte migration, selection, and signaling at the different stages of T-cell development.

T-cell development takes place in the thymus from progenitors that immigrate from the bone marrow, and during this process precursor thymocytes proliferate, rearrange their antigen receptor DNA, express functional TCRs and co-receptors, and undergo a series of selection events (Petrie and Zuniga-Pflucker, 2007). Intrathymic T-cell differentiation in the mouse can be divided into three major stages distinguished by the expression of TCR/CD3, CD4, and CD8 (Fig. 4.2). In the first major stage, neither the TCR, CD4, nor CD8 is expressed; most immunologists refer to this stage as the double negative (DN) or $CD4^-$ $CD8^-$ stage. It is during the third phase of this stage (DN3; see below) that rearrangement of the VDJ segments of the TCRβ,

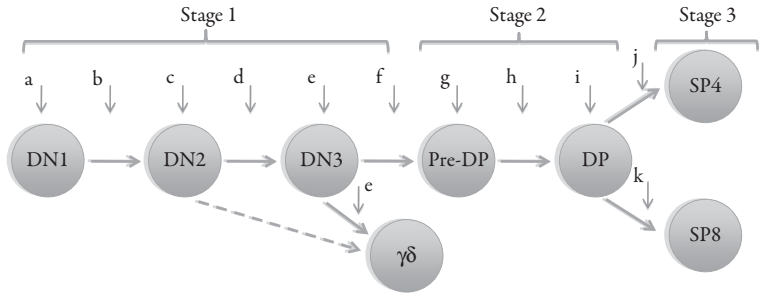

Figure 4.2 Stage-specific events, a brief overview of T-cell development. Stage 1: Double negative: a, DN1 thymocytes are heterogeneous; DN1 cells (CD44+ CD25-) expressing CD117hi CD24-/lo correspond to the ETP subset; b, Notch-1/Delta-like-4 interactions induce commitment to the T-cell lineage; c, TCR rearrangements are initiated by CD44+ CD25+ DN2 cells; d, Irreversible commitment to the T-cell lineage; e, αβ and γδ lineage diverge, due to TCRβ-selection and γδ-TCR signals at the CD44- CD25+ DN3 phase; f, Pre-TCR dependent survival, proliferation, and differentiation are induced. Stage 2: Double positive: g, TCRα rearrangements; CD4 and CD8 expression are initiated; h and i, Positive selection and negative selection take place. Stage 3: Single positive cells: j and k, Final lineage commitment dependent of the kinetic signaling model takes place.

TCRγ, and TCRδ (but not TCRα) loci is completed, and the TCRαβ and TCRγδ lineages diverge (Ciofani et al., 2006). In cells destined to become mature αβ T-cells, the TCRβ chain pairs with an invariant germline-encoded TCRα-surrogate chain known as the pre-Tα chain to form a pre-TCR complex on the cell surface (von Boehmer, 2005). This stage is known as the β-selection checkpoint, as thymocytes expressing nonfunctional TCRβ chains die at this point due to a lack of survival signaling (Michie and Zúñiga-Pflücker, 2002). In the second major stage of αβ T-cell development, thymocytes commence expression of both co-receptors and are known as double positive (DP) or $CD4^+$ $CD8^+$ thymocytes. Rearrangement of the TCRα locus occurs, expression of pre-Tα is extinguished, and true TCRαβ heterodimers are expressed on the surface of DP thymocytes. DP thymocytes then undergo positive selection to preserve cells binding to self-antigen/MHC with low to moderate affinity, followed by commitment to either the CD4 or CD8 lineage. The resulting single positive (SP) thymocytes undergo negative selection to remove autoreactive T-cells and emerge from the thymus as mature $CD4^+$ and $CD8^+$ αβ T-cells. These cells take up residence in the periphery, ready to detect foreign antigens. The development of γδ T-cells parallels the above course but differs from it in significant ways (see below).

The positive and negative selection events that occur during αβ T-cell development ensure that an optimal T-cell repertoire is generated. Negative selection refers to the deletion by induced apoptosis of potentially self-reactive thymocytes; that is, those cells that strongly recognize self-peptide/MHC (pMHC) presented by thymic stromal cells. Positive selection refers to the differentiation of thymocytes that only weakly recognize self-pMHC (those cells that are more likely to recognize a set of foreign peptides presented on self-MHC). Positive selection thus generates a T-cell repertoire that is restricted to self-MHC (Bevan, 1977; Zinkernagel et al., 1978; Zinkernagel and Doherty, 1979), and negative selection ensures that that repertoire is self-tolerant (Kappler et al., 1987a, 1987b, 1988; MacDonald et al., 1988). γδ T-cells also undergo TCR-mediated selection events, but the outcome of this selection process appears to be geared for ligand recognition, which results in the functional maturation of different subsets of γδ T-cells (Hayes and Love, 2007; Jensen and Chien, 2009). Taken together, immunologists call the outcome of these processes "the establishment of *central tolerance and self-MHC-restriction.*" T-cell clones that recognize self-antigens but escape deletion or inactivation in the thymus are functionally inactivated in the periphery by mechanisms of *peripheral tolerance*.

THYMOCYTE DIFFERENTIATION

With this background in T-cell development, we are ready to examine thymocyte differentiation in detail. As we follow hematopoietic precursor cells on their journey from lymphocyte progenitors to developing thymocytes to mature T-cells, we will at times make reference to the gene-targeted mice whose phenotypes have helped to unravel the functions of various genes involved in this process (Mak et al., 2001; Yeung et al., 1994). These mutant animals were of key importance in establishing that T-cells mature through multiple genetic checkpoints (Fig. 4.2). For example, deletion of the TCRβ locus leads to a earlier block in thymocyte development, during Stage 1, than the deletion of the TCRα locus (Mombaerts et al., 1992a), which results in a block at Stage 2. These results helped define the two distinct points during which products of TCR gene rearrangement become necessary for further thymocyte differentiation. The signaling consequences of TCRβ or TCRαβ expression were revealed by the phenotype of Lck-deficient mice (Molina et al., 1992), which were found to have the similar defects as TCRβ-deficient animals. Lck signaling function had not been expected to be important at so early a stage because it was generally assumed that the TCR was not expressed on early thymocytes. The door was thus opened to the discovery of the pre-TCR signaling complex and a key checkpoint in T lymphopoiesis (Michie and Zúñiga-Pflücker, 2002; von Boehmer et al., 1999).

T-CELL DEVELOPMENT

All hematopoietic cells, including T-cells, originate from hematopoietic stem cells present in the fetal liver during embryonic development and in the bone marrow in adulthood.

According to the most widely accepted model of murine hematopoiesis, the process starts with the hematopoietic stem cell (HSC), which is capable of indefinite self-renewal in vivo and can reconstitute the complete spectrum of hematopoietic cells. HSCs, resident in the adult bone marrow, differentiate to give rise to downstream progenitors, which include cells with multipotent progenitor (MPP) function that have short-term reconstitution ability, as well as lineage-restricted progenitors such as lymphoid-primed multipotent progenitors (LMPP) (Adolfsson et al., 2005). These $Flt3^+$ multipotent progenitors retain limited self-renewal capacity and are able to differentiate into the full complement of myeloid cells and lymphoid cells, but not erythroid or megakaryocytic cells lineages. Upon release from the bone marrow into the blood, some LMPPs give rise to lymphoid progenitors that commence expression of the chemokine receptors CCR7, CCR9, and CXCR4 (Benz and Bleul, 2005; Takahama, 2006), as well as other receptors that mediate the receipt of recruitment signals, a combination of chemokine ligands and adhesion molecules, emanating from the cortical side of venules at the corticomedullary junction (CMJ) of the thymus (Fig. 4.3). Upon entering and seeding the thymus, these cells become known as "early thymic progenitors" (ETPs) and belong to the DN1a phase of Stage 1 of T-cell development (Fig. 4.2). These cells are critical for thymic structure and function, as this organ does not contain its own self-renewing stem cell population and depends on continuous colonization by bone marrow-derived ETPs to maintain its ongoing task of producing T-cells (Goldschneider et al., 1986; Petrie and Zuniga-Pflucker, 2007). Several different types of ETPs capable of seeding the thymus and committing to the T lineage have been identified (Porritt et al., 2004), but, due to their scarcity, their further characterization remains under investigation (Bhandoola et al., 2007; Saran et al., 2010).

THYMIC STRUCTURE AND FUNCTION

The mouse thymus makes its first appearance in the embryo between day E10.5 and E11.5, when the third pharyngeal pouch endoderm interacts with surrounding mesenchyme to form the thymic anlagen (Blackburn and Manley, 2004). At this stage, the organ is composed largely of immature or progenitor thymic epithelial cells (TECs), which later generate the cortical and medullary TEC subsets that establish the framework of the mature cortex and medulla regions of the thymus (Rossi et al., 2006). Under the influence of the chemokine CCL25 secreted by TECs, the first ETPs (which express CCL25's receptor CCR9) enter the thymic primordium at about E12 and colonize it. TECs also express P-selectin, an adhesion molecule that can bind to its ligand PSGL-1 expressed on ETPs (Rossi et al., 2005). This binding may be significant for the establishment of progenitors in the thymus, as the thymi of mice lacking PSGL-1 show very few thymocytes. Once in the thymus, the ETPs interact with TECs and receive survival, proliferation, and differentiation signals delivered by cytokines, Flt3, SCF and IL-7, and Notch ligands, Delta-like-4 (Petrie and Zuniga-Pflucker, 2007). Conversely, the ETPs deliver signals to TECs that are needed for the structural organization of the mature thymus (Klug et al., 1998, 2002). Additionally, the formation of a mature medulla relies on signals mediated by

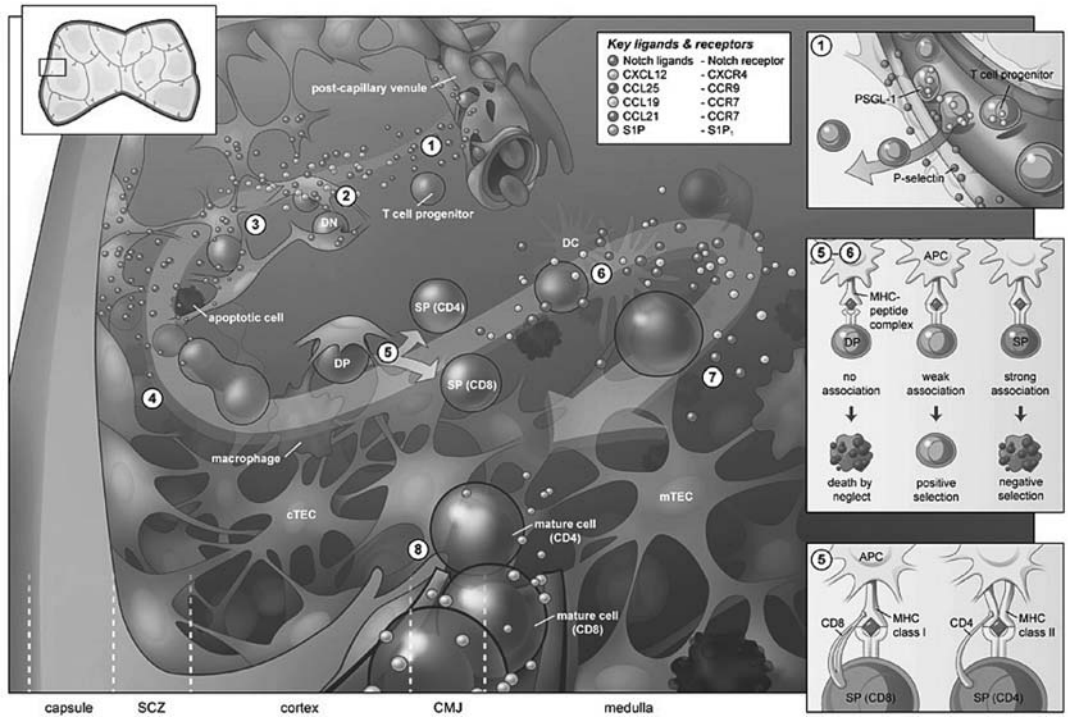

Figure 4.3 Intrathymic T-cell development, step by step. 1: Recruitment of T-cell progenitors; 2: Migration to cortex; 3: TCRβ locus rearrangement; 4: Rapid proliferation of post-β-selected DN cells, followed by TCRα rearrangement and CD4 and CD8 expression; 5: Positive selection, and CD4/CD8 lineage divergence; 6: CCR7-mediated migration to the medulla for negative selection; 7: Final maturation; and 8: Exportation of mature T-cells from the thymus. (See Color Plate.)

the TNF family member, RANKL, expressed by some thymocyte subsets (Hikosaka et al., 2008; Rossi et al., 2007). Several other molecules have been identified as being important for TEC development and/or function, such as FoxN1 (*Whn*), a transcription factor shown to be important for TEC development and the establishment of a normal thymic microenvironment (Blackburn and Manley, 2004; Nehls et al., 1994, 1996); see also Chapter 21.

The thymic microenvironment is absolutely crucial for the development of ETPs through all the stages of T-cell development (Fig. 4.3). *Nude* mice, which lack a thymus and mature TECs due to loss of function of the transcription factor FoxN1 (Blackburn and Manley, 2004; Nehls et al., 1994, 1996), also lack all T-cells. Moreover, specific regions of the thymus foster the progression of different stages of thymocyte development, giving rise to a complex migratory pattern of thymocytes within this organ (Takahama, 2006).

Step by Step, Moving Through the Thymus

In an adult thymus, thymus-seeding cells, which resemble LMPPs, exit the blood and arrive at the thymic CMJ are deemed to be DN1/ETPs thymocytes (refer to Fig. 4.3). With the support of cortical TECs (cTECs), the DN1 cells give rise to DN2 thymocytes and migrate into the center of the cortex, where commitment to the T lineage occurs (a process that can consume up to 2 weeks). The resulting DN3 thymocytes migrate further into the cortex and enter the subcapsular zone (SCZ), where the process of β-selection takes place (Fig. 4.3). The DN4 or pre-DP thymocytes emerging from this culling differentiate further into intermediate single positive (ISP) cells while still in the SCZ and outer cortex, but then migrate back through to the central cortex as they become DP thymocytes. The DP cells in the cortex encounter pMHC complexes presented by cTECs and undergo positive selection to ensure the survival of T-cells that recognize self-MHC. CD4/CD8 lineage commitment to generate early SP cells of both subsets then follows. These SP cells migrate out of the cortex into the medulla, coming into contact with medullary TECs (mTECs) and thymic DCs that enforce negative selection. The SP thymocytes that survive negative selection exit the medulla and enter the blood, becoming new and mature naïve T-cells that will home to the lymphoid organs (Petrie and Zuniga-Pflucker, 2007; Takahama, 2006).

Transcription Factors

The transcription factor PU.1 controls the expression of many cytokine receptors and thus controls the delivery of developmental signaling mediated by the corresponding cytokines (Rothenberg, 2007). In particular, PU.1 is required for the transcription of the IL-7 receptor (IL-7R) subunits needed to receive IL-7 signaling (see below). PU.1$^{-/-}$ mice lack not only T-cells but also B-cells, neutrophils, and macrophages (Singh et al., 1999). The level of PU.1 in a hematopoietic progenitor controls its destiny: low levels of PU.1 induce IL-7R expression and lymphoid development, whereas high levels of PU.1 suppress IL-7R expression and promote the expression of receptors for cytokines favoring myeloid development.

Ikaros is a transcription factor acting slightly later than PU.1 that is also required for lymphoid progenitor development (Rothenberg, 2007). Ikaros is thought to repress the transcription of "lineage-inappropriate" genes by recruiting histone deacetylases that reduce the chromatin accessibility of a locus (Georgopoulos, 2002). For example, CD19 (a B-cell-specific gene) is repressed in progenitors destined to become T-cells, whereas expression of CD4 and CD8 is repressed in precursors destined to become B-cells. A related transcription factor called Aiolos interacts with Ikaros to help establish T-lineage commitment and thymocyte expansion. Mice with reduced levels of Ikaros or Aiolos fail to later activate expression of the CD8α gene, leading to an apparent increase in immature T-cells expressing CD4. Another important transcription factor involved in the divergence of T-cell progenitors from B-cell progenitors is GATA-3 (Pai et al., 2003). GATA3$^{-/-}$ mice can produce B-cells but not even the earliest of thymocyte populations (Hare et al., 1999).

Cell-Fate Molecules

The Notch signaling pathway plays a role in almost all binary cell-fate decisions in a very wide range of organisms (Artavanis-Tsakonas et al., 1999). Interactions between the four mammalian Notch receptors (Notch1–4) and their five ligands (Jagged-1, Jagged-2, Delta-like-1, Delta-like-3, and Delta-like-4) mediate signaling between neighboring cells that leads to the regulation of transcription via direct association with transcription factors (Bray, 2006). Engagement of a Notch receptor by ligand results in proteolytic cleavage of the Notch protein by ADAM proteases and the release of Notch's intracellular domain (Notch-ICD). Of all the Notch receptors and ligands, only Notch-1 interaction with Delta-like ligands is involved in T-lineage specification (Jaleco et al., 2001), and continuous Notch-1 signaling is crucial for murine T-cell development from the earliest DN1 cells through to the DN3 stage (Schmitt et al., 2004). Indeed, a major lymphopoietic function of the thymic microenvironment is to supply ligands, in particular Delta-like-4 being the relevant ligands expressed by TECs (Hozumi et al., 2008; Koch et al., 2008), for Notch signaling (Radtke et al., 2004; Zuniga-Pflucker, 2004). Nevertheless, ectopic expression of Delta-like-1 by bone marrow stromal cell lines induces the differentiation of hematopoietic progenitors into αβ and γδ T-cells but not B-cells (Jaleco et al., 2001; Schmitt and Zuniga-Pflucker, 2002). Conversely, loss of Delta-like-4 expression in fresh ex vivo thymic stromal monolayers prevents them from supporting T-lineage development (Mohtashami and Zuniga-Pflucker, 2006).

In the absence of Notch-1 signaling, V to DJ recombination in the TCRβ locus is impaired and murine thymocyte development is halted at this stage (Wolfer et al., 2002). Moreover, rather than T-lineage cells, progenitors of NK cells or plasmacytoid dendritic cells (pDCs) may appear (Dontje et al., 2006; Schmitt et al., 2004). Notch-1 also interferes with the activity of the E2A transcription factor that facilitates transcription of B-cell-specific genes, blocking development down the B-cell path (Ikawa et al., 2006). Consistent with these findings, loss-of-function mutations of Notch-1 in newborn mice or in bone marrow stem cells result in a severe early block in T-cell

development and the appearance of ectopic B-cells in the thymus (Han et al., 2002; Koch et al., 2008; Radtke et al., 1999; Wilson et al., 2001). In addition, Notch-1 function can be manipulated to favor the development of B-cells in the thymus (Izon et al., 2002; Koch et al., 2001). Conversely, expression of a constitutively active form of Notch-1 in bone marrow stem cells results in the ectopic development of precursor T-cells outside the thymus (Allman et al., 2001; Pui et al., 1999).

Upon Notch-1 cleavage, Notch-ICD translocates to the nucleus and interacts with the RBPJ transcription factor, converting it from a repressor to an activator of genes involved in cell-fate decisions (Hsieh et al., 1996; Struhl and Adachi, 1998). Among the cell-fate genes influenced by Notch-ICD are the HES genes, which encode transcriptional repressors that act as downstream effectors in the Notch-1 signaling pathway. $HES^{-/-}$ mice have a small thymus, which is deficient in mature αβ and γδ T-cells (B and NK cells are normal). The major effect of HES may be on T-lineage progenitor survival and proliferation, as overexpression of Hes-1 in thymocytes enhances their growth (Kaneta et al., 2000) but depresses B-lineage cell numbers (Kawamata et al., 2002). Notch-ICD also recruits transcriptional co-activators of the "mastermind-like" (MamL) family (Bray, 2006). Overexpression of dominant negative (DN) MamL in hematopoietic progenitors results in aberrant lymphopoiesis in the thymus (Maillard et al., 2004). More recently, it has been demonstrated that murine ETPs lacking Mint, an inhibitor of Notch-1-mediated transcription, remain stuck at the DN1 stage (Tsuji et al., 2007), suggesting that the levels of Notch signaling must be appropriately coordinated to ensure proper induction of T-lineage commitment and differentiation.

Cytokines

Stem cell factor (SCF; also known as c-kit ligand) is a key cytokine that binds to c-kit tyrosine kinase receptor (CD117) and triggers signaling supporting the earliest stages of T lymphopoiesis (Rodewald et al., 1995). SCF is produced by TECs but the precise location of these cells in the thymus remains uncertain. SCF, which is required for the expansion of DN1 and DN2 cells but not DN3 cells (Massa et al., 2006), appears to maintain early T-lineage cells in a relatively undifferentiated state. SCF works in concert with low levels of IL-7 to promote the differentiation of HSCs into myriad cell lineages, including T-cells (Rodewald et al., 1997).

Morphogens

Morphogens are secreted molecules that regulate tissue patterning and organogenesis during embryogenesis. Important morphogens are members of the Wnt, Hedgehog, and bone morphogenetic protein (BMP) families. Although these molecules act as cell-fate specifiers in many tissues, they are not involved in this capacity in T-cell development. Rather, morphogens secreted by both thymic stromal cells and thymocytes themselves regulate survival and proliferation. In particular, Wnt is required for regulation of FoxN1 expression in TEC cell lines (Balciunaite et al., 2002) and for proliferative signals during the DN1–DN3 stages (Weerkamp et al., 2006). However, the involvement of the Wnt downstream target β-catenin during T-cell development remains under investigation (Cobas et al., 2004; Xu et al., 2003, 2009). The hedgehog morphogens are also expressed at high levels from the ETP stage until pre-TCR signaling during the DN3 stage (El Andaloussi et al., 2006). Mice deficient for sonic hedgehog have a thymus of reduced cellularity due to defects in DN1 and DN2 thymocyte proliferation (Shah et al., 2004). The BMPs appear to act as brakes on T-cell differentiation. BMP7 is expressed by thymocytes and thymic stromal cells, whereas BMP2 and BMP4 are expressed by stromal cells in the thymic medulla and SCZ (Graf et al., 2002; Hager-Theodorides et al., 2002). Signaling by these molecules is essential for the development of the thymic stroma (Bleul and Boehm, 2005) and modulates the expression of FoxN1 and chemokines by stromal cells (Tsai et al., 2003). With respect to T-cells, BMP2 and BMP4 promote the survival of DN1 cells but inhibit their proliferation and differentiation, and also block the DN4-to-DP transition.

THE DN1 STAGE

The earliest recognizable T-cell precursors belong to the DN1 subset (Figs. 4.2 and 4.3), which lack a TCR complex and co-receptors (and so are characterized as TCR/$CD3^{-}CD4^{-}CD8^{-}$), and have the following phenotype $CD117^{+}CD44^{+}CD25^{-}CD24^{-/lo}$ (Porritt et al., 2004). These cells, which constitute approximately 2 to 3 percent of all thymocytes, have the ability to home directly to the thymus after intravenous transfer into a host animal (Shortman, 1992). Entry of these cells into the thymus is facilitated by their expression of CCR7 and CCR9 chemokine receptors (Zlotoff et al., 2010), along with PSGL-1 (Rossi et al., 2005), which ensure homing and adhesion, respectively. DN1 cells typically reside in the thymic perimedullary cortex (PMC; the narrow band of cortex adjacent to the medulla) for about 10 days, during which time they proliferate vigorously and increase their numbers about 1,000-fold (Shortman et al., 1990). DN1 thymocytes have the differentiation potential to give rise to both αβ and γδ T-lineage cells, as well as other thymus-derived lineages such as certain DCs and NK cells (Bhandoola et al., 2007). DN1 cells subsequently upregulate expression of CD25 to become DN2 cells, and commence migration toward the outer thymic cortex under the influence of CXCL12, CCL19, and CCL21 produced by the cTECs. As pointed out above, Notch signaling plays an instructive role at this stage of differentiation, in which Notch-1 expressed on the DN1 cells engages Delta-like-4 on the cTECs to initiate the induction of T-cell lineage commitment (Rothenberg et al., 2008).

THE DN2 STAGE

DN2 cells show a surface phenotype of $CD117^{+}CD44^{+}CD25^{+}CD24^{hi}$. DN2 cells are also highly proliferative but have a more limited differentiation potential than DN1 cells, so that the T-precursor cell population expands (Petrie and Zuniga-Pflucker, 2007). Although early DN2 cells retain some capacity to develop into NK cells and certain DC

lineages, cTECs continue to deliver strong Notch signals favoring T-lineage commitment and differentiation (Schmitt et al., 2004). DN2 cells reside in the inner cortex and remain there for about 2 days (Porritt et al., 2003), while αβ:γδ lineage divergence commences due to activation of the RAG genes, which will lead to the rearrangement and generation of the TCRs that will ultimately influence the lineage choice. The DN2 cells start to express the pre-Tα chain in preparation for β-selection. The majority of DN2 cells retain potential to give rise to both αβ and γδ lineages, while some DN2 cells with early γδ TCR expression are committed to this lineage (Ciofani et al., 2006).

Cytokines

Although ETP/DN1 cells express only low levels of IL-7Rα (Allman et al., 2003; Porritt et al., 2004), IL-7 (in combination with Flt3-ligand and SCF, all expressed by TECs) is essential for the survival and maturation of the IL-7Rα-expressing DN2 and DN3 cells that follow them. The stromal cells producing this IL-7 are located mainly in the medulla and CMJ of the thymus (Griffith et al., 2009; Zamisch et al., 2005). Mice with disruptions of the genes encoding either IL-7 or the IL-7R subunits have defects in cell-cycle progression and exhibit an abrupt block in thymocyte development at the DN2 stage. If a transgene encoding the anti-apoptotic protein Bcl-2 is introduced into mice deficient in IL-7 or IL-7R, T-cell development can be rescued (Akashi et al., 1997; Maraskovsky et al., 1997; von Freeden-Jeffry et al., 1995). This result implies that a primary function of IL-7 is to maintain thymocyte survival, which is likely mediated by controlling the expression of the Bcl2-related gene, Mcl-1 (Opferman et al., 2003). Interestingly, in addition to its role in preventing apoptosis, signaling delivered through IL-7R is also important for γδ T-cell development. IL-7R$^{-/-}$ mice have decreased numbers of αβ T-cells but a complete absence of γδ T-cells due to a failure in TCRγ locus rearrangement (Laky et al., 2003; Maki et al., 1996). Introduction of an already rearranged TCRγ transgene can rescue γδ T-cell development in these animals (Kang et al., 1999). It was originally thought that signals initiated by IL-7 binding to IL-7R and transduced via the intermediaries Jak and STAT triggered changes to the accessibility of the TCRγ locus that are required for RAG recombinase binding (Schlissel et al., 2000). However, IL-7-deficient mice expressing a γδ TCR transgene still have a defect in T-cell development, implying that IL-7 continues to deliver essential survival signals to γδ T-cells (Laky et al., 2003).

Chemokines

The migration of DN1/ETP cells into the inner cortex to generate DN2 cells is essential for successful T-cell development, and the chemokine CXCL12 may drive this process. Late DN1 thymocytes express CXCR4, which binds to CXCL12 that is expressed by cTECs in the inner cortex (but not by mTECs) (Griffith et al., 2009). Interestingly, CXCL12-expressing cells are scattered throughout the inner cortex, so that recruitment is likely mediated by forces other than a concentration gradient (Pelletier et al., 2000).

THE DN3 STAGE

As DN2 thymocytes give rise to DN3 cells, they downregulate expression of CD117 and CD44 to become CD117$^{-/lo}$CD44$^{-/lo}$CD25^{+} cells (Figs. 4.2 and 4.3). Early DN3 cells reside in the outer cortex and stay in this location for about 2 days before moving to the SCZ and spending another day there (Porritt et al., 2003). It is at the DN3 stage that the final commitment to the αβ or γδ T lineage is made (Ciofani et al., 2006). Although both the αβ and γδ T lineages arise from the same DN progenitors, it is still not absolutely clear how the commitment decision is made due to the difficulty in distinguishing between these cells prior to TCR expression. However, high levels of IL-7R, along with Sox13, expression by DN2 cells may predispose them to adopt the γδ T-cell lineage (Kang et al., 2001; Melichar et al., 2007). Analyses of DN2 cells have indicated that lineage divergence commences at this point and is completed at the DN3 stage, depending on whether a pre-TCR (pTα/TCRβ) or a functional γδ TCR has been expressed on the cell surface (Ciofani et al., 2006). Thus, thymocytes at the DN2 to DN3 stages undergo RAG-mediated somatic recombination at the TCRβ, γ, and δ gene loci (Capone et al., 1998; Livak et al., 1999; Raulet et al., 1985). While only rearrangement of the TCRβ locus is required to initiate αβ T-cell maturation, both TCRγ and TCRδ must be productively rearranged to generate functional γδ T-cells. These is also strong evidence that the strength of the signal delivered via the pre-TCR or γδ TCR solidifies or mediates the commitment decision undertaken by DN2/DN3 thymocytes (Haks et al., 2005; Hayes et al., 2005; Kreslavsky et al., 2008).

Transcription Factors

Sox13 is a transcription factor that has been closely associated with DN2 cell precommitment and αβ:γδ lineage divergence (Melichar et al., 2007). In mice, deficiency for Sox13 impairs γδ T-cell development. Sox13 expression is highest among DN2 cells and drops off dramatically by the DN3 stage. Recently, it was shown that the differential activation of the extracellular signal-related kinase (ERK)-early growth response gene (Egr)-inhibitor of DNA binding 3 (Id3) pathway plays a defining role in mediated strong TCR signals leading to γδ-lineage commitment (Lauritsen et al., 2009). In particular, Id3 expression, a negative regulator of E2A function, served to regulate adoption of the γδ fate.

Cell-Fate Molecules

Expression of Notch-1 and Notch-3 is highest in DN3 cells (Taghon et al., 2006), consistent with an important role for these receptors in the αβ:γδ lineage decision. However, rather than driving a differentiation direction, Notch molecules appear instead to promote the survival and expansion of preselection DN3 thymocytes, and then are required only for the further differentiation of β-selected αβ-lineage cells (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005, 2007). The key survival kinase Akt remains activated in DN3 cells in response to Notch signaling, and glucose and protein metabolism are bolstered in preparation for the energy-intensive events of αβ:γδ divergence.

Enzymes and Signaling Molecules

Because RAG1 and RAG2 are essential for the rearrangement of all TCR genes (Schatz et al., 1989), it is not surprising that mice deficient for either of these molecules have early defects in lymphocyte development (Mombaerts et al., 1992b; Shinkai et al., 1992, 1993). The thymi of RAG1- or RAG2-deficient mice contain a decreased number of total thymocytes (approximately 1 to 3×10^6 compared to about 1×10^8 thymocytes in normal mice), and T-cell development is arrested at the DN3 stage (Michie and Zúñiga-Pflücker, 2002). Similarly, mice with a natural mutation in the dsDNA-PK (*Prkdc*) gene show normal initiation of gene segment recombination but fail to ligate the segments together, resulting in a lack of mature T and B-cells (Kirchgessner et al., 1995). In contrast, mice with a mutation in the *Tdt* gene responsible for N-nucleotide addition do not show altered T-cell development (Gilfillan et al., 1993; Komori et al., 1993).

DN3 Thymocytes and β-Selection

In DN3 thymocytes destined to become αβ T-cells, the TCRα locus does not initiate rearrangement until considerably later than the other three loci. Instead, newly synthesized TCRβ chains pair with pre-Tα chains to form the pre-TCR. Pre-TCRs undergo testing to determine whether the TCRβ chain can function in a signaling complex (Michie and Zúñiga-Pflücker, 2002). Only DN3 pre-T-cells that have rearranged the TCRβ locus in-frame and have successfully combined it with the pre-Tα chain to form a functional pre-TCR on the thymocyte surface receive a signal permitting further development, a process called "β-selection" (Dudley et al., 1994; Hoffman et al., 1996). Within DN3 cells that are β-selected, further rearrangements of the TCRβ locus on both chromosomes are shut down, a process known as "allelic exclusion."

The survival signal associated with β-selection is mediated by activation of the transcription factor NF-κB, which controls the expression of numerous genes needed for staving off cell death and inducing proliferation (Voll et al., 2000). Cells that have rearranged the TCRβ locus out-of-frame do not receive the survival signal and are induced to undergo apoptosis (Malissen and Malissen, 1996). Thus, disruption of the pre-T gene leads to an arrest in TCRαβ thymocyte development at a phenotypic stage similar to that in TCR-deficient mice (Fehling et al., 1995). TCR-deficient mice have a small thymus due to an early block in T-cell development at the DN3 stage. The pTα/TCRβ complex undergoes self-oligomerization and assembles with the CD3 complex in the plasma membrane microdomains to deliver a cell-autonomous signal that "instructs" T precursors to divert down the αβ differentiation path (O'Shea et al., 1997; Yamasaki et al., 2006). Interestingly, pre-T-deficient mice exhibit normal development of γδ T-cells, indicating that the γδ T lineage diverges from the αβ T lineage at the point of pre-TCR expression (Fehling et al., 1995; Malissen and Malissen, 1996; Michie and Zúñiga-Pflücker, 2002).

In addition to the pre-Tα chain, pre-TCR signaling requires intact function of components of the CD3 complex, signal transduction molecules and adaptor molecules further downstream. For example, $CD3^{-/-}$ thymocytes, although still able to express rearranged TCRβ transcripts, are blocked at the DN3 stage (Malissen et al., 1995). These findings imply that CD3 expression is important for monitoring the productivity of TCR rearrangement by testing for pre-TCR signal transduction. Consistent with the association between pre-TCR signaling and αβ T commitment, rearrangements of TCR (*Tcrg*) and (*Tcrd*) gene segments are normal in $CD3^{-/-}$ mice. Mice deficient for CD3ζ show a profound reduction in the amount of surface TCRs on DP cells as well as a reduced number of thymocytes (Liu et al., 1993; Love et al., 1993; Malissen et al., 1993; Ohno et al., 1993; Shores et al., 1998). In the peripheral lymphoid organs of double mutant $CD3^{-/-}$ mice, abnormal $CD4^+CD8^-TCR\alpha\beta^+$ and $CD4^+CD8^+TCR\alpha\beta^+$ cells were present. Interestingly, the development of thymus-independent intestinal intraepithelial lymphocytes (IELs) occurred normally in these mice, and these IELs expressed TCRs complexed with the $Fc_\varepsilon RI_\gamma$ chain in the absence of CD3ζη (Love et al., 1993; Malissen et al., 1993).

Enzymes and Signaling Molecules

Lck kinase is important for β-selection. Mice either deficient for Lck kinase (Molina et al., 1992) or transgenic for a dominant-negative Lck mutation (Levin et al., 1993) exhibit a small thymus due to an early block in the maturation of both TCRαβ and TCRγδ thymocytes. Indeed, a transgene encoding a constitutively active form of Lck can fully replace defective pre-TCR signaling in RAG1-deficient mice (Mombaerts et al., 1994). Thus, the catalytic activity of Lck is crucial for the maturation of early β- and γδ-selected thymocytes. These results were confirmed using RAG complementation studies in which Lck was shown to be critical for signaling pathways activated by γ-irradiation and CD3ε engagement in $RAG\text{-}1^{-/-}$ immature thymocytes (Wu et al., 1996). However, a loss of Lck function during early thymocyte development and pre-TCR signal transduction can be compensated for by Fyn kinase. Mice deficient for Fyn kinase alone show no overt phenotypic changes in T-cell development (Appleby et al., 1992; Stein et al., 1992), but double Lck/Fyn-deficient mutants have a block at the DN3 stage that is more severe than that observed in Lck-deficient animals (Groves et al., 1996; Van Oers et al., 1996). Several other signaling molecules have been shown to be crucial at the DN3 stage for pre-TCR signaling and DN3 thymocyte maturation along the αβ-lineage, and these have been previously reviewed (Aifantis et al., 2006; Kruisbeek et al., 2000; Michie and Zúñiga-Pflücker, 2002).

Morphogens

Wnt signaling is crucial for the further maturation of αβ DN3 thymocytes (Fukushima et al., 2006; Gounari et al., 2001) but, interestingly, not for γδ DN3 thymocytes (Xu et al., 2003). There is some evidence suggesting that differential Wnt signaling may contribute to the αβ versus γδ lineage decision, since Sox13, which is specific regulator of γδ T-cell

differentiation, may antagonize downstream Wnt effectors (Melichar et al., 2007).

Chemokines

Early DN3 cells in the outer cortex are thought to be influenced by stromal cell-produced CCL25, which binds to CCR9. DN3 cells must express CCR9 to receive signals directing them to the SCZ prior to β-selection (Benz et al., 2004), and overexpression of CCR9 leads to an accumulation of DN3 cells (Uehara et al., 2002). CXCL12, which binds to CXCR4, is also important for DN3 cell migration within the cortex (Plotkin et al., 2003).

γδ DN3 Thymocytes

Murine γδ DN3 thymocytes are $CD25^-CD4^-CD8^-$ but express a complete γδ TCR rather than a molecule analogous to the pre-TCR of αβ DN3 thymocytes. Although this γδ TCR does not associate with either lipid microdomains in the plasma membrane or CD3ε (Saint-Ruf et al., 2000), it mediates the selection γδ DN3 thymocytes. The ligand(s) involved remain unclear (Jensen and Chien, 2009). However, studies employing a green fluorescent protein (GFP) reporter of TCRδ locus transcription in combination with γδ TCR surface expression showed that γδ TCR signaling is necessary for the survival and expansion of γδ DN3 thymocytes that eventually give rise to mature γδ T-cells (Prinz et al., 2006). These β-selection and γδ-selection processes are apparently independent, as mice with a targeted disruption of the TCR Cδ exon have no detectable cell surface expression of any γδ TCR components but display normal αβ T-cell development (Itohara et al., 1993). TCRδ-deficient mice are also able to mount normal antibody responses after immunization with ovalbumin, indicating that αβ T-helper cell function remains intact (Itohara et al., 1993). Conversely, development of γδ cells is unaffected by either *Tcra* or *Tcrb* mutations (Mombaerts et al., 1992a). The independence of the αβ versus γδ lineages is emphasized by the observation that γδ DN3 thymocytes exhibit a gene expression profile that is distinct from that of αβ DN3 thymocytes (Pennington et al., 2003, 2005; Silva-Santos et al., 2005). However, there is clear evidence that the two lineages influence each other's development in subtle ways, as γδ DN3 thymocytes developing in pre-TCR-deficient mice generate atypical γδ T-cells (Pennington et al., 2003; Silva-Santos et al., 2005).

Enzymes and Signaling Molecules

Lck kinase is also involved in the development of γδ DN3 thymocytes. The maturation of transgenic TCRγδ T-cells was followed in $Lck^{-/-}$ mice and found to be blocked at an early stage (Penninger et al., 1993). However, the development of these transgenic γδ T-cells did not require the co-expression of CD4 or CD8, indicating that the Lck/CD4 and Lck/CD8 associations normally observed in wild-type αβ T-cells are not essential for γδ T-cell development (Penninger et al., 1993). Surprisingly, as was true for CD3ζη-deficient mice, intestinal TCRγδ T-cells appeared to be unaffected by the Lck mutation (Penninger et al., 1993).

αβ DN4 OR PRE-DP THYMOCYTES AND THE IMMATURE SINGLE POSITIVE (ISP) STAGE

As β-selected murine DN3 thymocytes further evolve into DN4 cells or per-DP cells, expression of CD25 is lost and the cells become phenotypically $CD44^-CD25^-$. These cells undergo a dramatic expansion that requires the activities of the many transcription factors (e.g., Tcf-1, c-myc, and Notch; Ciofani and Zuniga-Pflucker, 2006; Douglas et al., 2001; Schilham and Clevers, 1998). DN4 cells that expand successfully then pass through the ISP stage, in which CD8 is transiently expressed on the cell surface but a mature TCR is not yet present (Nikolic-Zugic and Bevan, 1988; Nikolic-Zugic et al., 1989; Petrie et al., 1990).

Cell-Fate Molecules and Morphogens

Notch-1 signaling has been shown to be essential for the DN-to-DP transition, but once fully differentiated DP thymocytes express low levels of Notch-1 (Ciofani and Zuniga-Pflucker, 2006). In contrast, the morphogen sonic hedgehog acts on DN thymocytes to hold them at this stage of development (Outram et al., 2000). The transition to DP cells cannot occur until an unknown mechanism blocks sonic hedgehog signaling by thymic stromal cells.

Cytokines

DN3 thymocytes start to downregulate their expression of IL-7R after β-selection so that DN4 cells are refractory to IL-7 signaling, a state maintained into the DP stage that follows (Van De Wiele et al., 2004; Yu et al., 2006).

THE DP STAGE

By definition, DP thymocytes are those that express both CD4 and CD8. In the mouse, these cells reside throughout the thymic cortex for about 2 to 3 days (Egerton et al., 1990). It is thought that gene expression programs differ between the DN thymocytes resident in the cortex and the DP cells resident in the cortex, such that the stromal signals governing DN cells are not received by DP cells and vice versa (Petrie and Zuniga-Pflucker, 2007). In DP cells destined to become mature αβ T-cells, rearrangement of the TCRα locus occurs, and a complete TCRαβ heterodimer is finally expressed on the thymocyte surface. These DP cells then decrease their proliferation in a cell-intrinsic manner (Petrie, 2003) and are subjected to positive selection and CD4/CD8 lineage commitment as described below.

Co-receptor Expression

$CD4^+CD8^+$ DP thymocytes rearrange their TCRα (*Tcra*) locus to generate a functional TCRαβ heterodimer (Petrie et al., 1993). Mice with a disruption in the TCRα locus consequently show a later block in development than do TCRβ-deficient mice. TCRα-deficient animals have a normal-sized thymus and a marked absence only of $CD4^+$ and $CD8^+$ SP cells (Mombaerts et al., 1992a; Philpott et al., 1992). Interestingly, normal numbers of both DN and DP

thymocytes expressing TCRβ chains but not heterodimeric αβ TCRs exist in TCR$\alpha^{-/-}$ animals. In older TCRα-deficient mice, an unusual CD4^{+}TCRβ^{dull} lymphocyte population can be observed in the periphery that is even more apparent after the introduction of a TCRβ transgene (Philpott et al., 1992). These data indicate that, while TCRα is not required for the DN-to-DP transition and the subsequent expansion of the DP thymocyte pool, its expression (along with that of TCRβ) is required for the DP-to-SP transition. The TCRα locus does not undergo allelic exclusion like the TCRβ locus and $V_{\alpha}J_{\alpha}$ rearrangements may continue within a given cell (Petrie et al., 1993), such that two TCRs containing different TCRα chains may be expressed on the surface (Heath et al., 1995). More recently, it has been noted that the progression of $V_{\alpha}J_{\alpha}$ rearrangement is controlled by thymocyte survival in a way that regulates the T-cell repertoire (Guo et al., 2002).

As might be expected, CD4 and CD8 are crucial for DP thymocyte maturation (Zuniga-Pflucker et al., 1991). The CD8α chain is required for the surface expression of both CD8α and CD8β. Thus, in mice with a disrupted *Cd8a* gene, there is a total absence of CD8 expression on the T-cell surface (Fung-Leung et al., 1991, 1994). The vast majority of T-cells in the periphery of these mutants are CD4^{+} T-cells whose maturation appears normal. Cytotoxic responses to alloantigens are partly impaired whereas cytotoxic responses to virus infections are severely inhibited. Studies of CD8$\alpha^{-/-}$ mice transgenic for a CD8α chain bearing point mutations of the Lck binding domain showed that the Lck-associative function of CD8 is not essential for thymic selection (Chan et al., 1994a).

In mice with a disrupted *Cd8b* gene, the CD8α^{+} T-cell population in the thymus and in most peripheral lymphoid organs is reduced to 20 to 30 percent of normal, and CD8α expression on thymocytes is decreased to approximately 50 percent of normal (Fung-Leung et al., 1994). This defect does not extend to CD8αα-expressing IELs, which develop extrathymically (Guy-Grand et al., 1991). Thus, CD8 is important only for thymically derived CD8^{+} T-cells. The absence of CD8β reduces but does not completely abolish thymic maturation of CD8^{+} T-cells. Moreover, peripheral T-cells in CD8$^{-/-}$ mice display effective cytotoxic activity against virus infections, suggesting that CD8β is not essential for cytotoxic effector functions of CD8^{+} T-cells (Fung-Leung et al., 1994).

Mice with a disruption in the *Cd4* gene have markedly decreased T-helper cell activity but normal development and function of CD8^{+} T-cells. Thus, the expression of CD4 on progenitor cells and on DP thymocytes is not obligatory for the differentiation of CD8^{+} cytotoxic effectors (Killeen et al., 1993; Rahemtulla et al., 1991). Curiously, CD4$^{-/-}$ mice are still able to mount detectable T-helper cell responses. This residual T-helper activity has been attributed to a population of TCRαβ^{+}CD4–8^{-} T-cells present in peripheral lymphatic organs (Locksley et al., 1993; Rahemtulla et al., 1994).

Overview of Thymic Selection

Positive selection of murine DP thymocytes typically leads to the generation of mature CD4^{+} TCR^{high} helper or CD8^{+} TCR^{high} cytotoxic SP T-cells that are able to react to non-self while self-tolerant and self-MHC restricted (Starr et al., 2003). Negative selection of these SP populations then eliminates potentially harmful self-reactive T-cells (von Boehmer et al., 1989). The mechanisms governing thymic selection are dependent on physical interactions between the TCRs expressed on developing thymocytes and the pMHC complexes expressed on surrounding thymic stromal cells (such as TECs and thymic DCs). Studies of most animal models of thymic selection support an affinity/avidity mechanism for this process, in which the type of selection a thymocyte undergoes depends on the strength of the TCR–pMHC interaction (Starr et al., 2003). According to this model, thymocytes with TCRs of low affinity/avidity for self-MHC (regardless of the peptide that MHC has bound) do not receive any type of signal and undergo apoptosis by default. More than 80 percent of developing T-cells fall into this category. However, thymocytes expressing TCRs with intermediate affinity/avidity for self-MHC receive a survival signal and are positively selected (Starr et al., 2003). These TCRs are those most likely to recognize foreign peptides presented in the context of self-MHC and thus may be useful in host defense against pathogen infections. In contrast, thymocytes expressing TCRs with high affinity/avidity for self-peptide/self-MHC receive a signal that induces them to undergo either clonal deletion or clonal inactivation. This negative selection of thymocyte clones removes potentially self-reactive cells. The affinity/avidity model has been tested using fetal thymic organ culture in which the density of specific pMHC complexes has been titrated against specific outcome. A low concentration of the peptide antigen recognized by a transgenic TCR resulted in positive selection, whereas a high concentration of the peptide antigen resulted in negative selection and immunological tolerance (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994; Starr et al., 2003).

Positive Thymic Selection

Murine DP thymocytes entering the inner cortex encounter cTECs that present self-MHC molecules loaded with peptides from a broad range of self-determinants (Starr et al., 2003). DP cells whose TCRs bind to these pMHCs with moderate affinity receive a survival signal, whereas cells whose TCRs fail to recognize self-MHC do not receive such a signal and are said to "die of neglect."

Anti-apoptotic and Transcription Factors

Studies of transgenic and knockout mice have shown that the genes encoding the anti-apoptotic protein Bcl-X_L, RhoA GTPase, the transcription factors NFAT and c-Myb, the retinoid-related orphan receptor (RORγ), and members of the E family of regulatory proteins are important for DP thymocyte survival (Ciofani and Zuniga-Pflucker, 2006). Similarly, studies of mice transgenic for mutant NF-κB regulatory proteins that act as inhibitors have shown that this transcription factor is crucial for positive selection.

Enzymes and Signaling Molecules

Dominant-negative transgenes have been used to inhibit various molecules within the mitogen-activated protein kinase

(MAPK) pathways important for signaling downstream of the TCR, particularly extracellular signal-regulated kinase (ERK) (Alberola-Ila et al., 1995b, 1996; O'Shea et al., 1996; Swan et al., 1995). Positive selection is blocked in mutants lacking ERK function, leading to a significant reduction in mature SP thymocytes. These results have been confirmed in mice genetically deficient for ERK1 (Pages et al., 1999) or its upstream mediator RasGRP (Dower et al., 2000). Other evidence suggests that the level of ERK signaling as induced by TCR affinity/avidity for the pMHC complex in question determines positive versus negative selection (Bommhardt et al., 2000; Hogquist, 2001; Mariathasan et al., 2000, 2001). In situations of negative thymocyte selection, ERK activation is vigorous but short-lived, whereas positive selection generates weak ERK signaling that is sustained (Mariathasan et al., 2001; Werlen et al., 2000). In contrast to the MAPK pathways, the phospholipid- signaling pathway that is activated in mature T-cells following TCR engagement does not appear to be important for thymic selection of DP cells. Initial analysis of mice deficient for protein kinase C theta (PKCθ) suggested that this kinase does not play an essential role in either positive or negative selection (Sun et al., 2000); however, it plays an important role in TCR signaling (Marsland and Kopf, 2008). It also remains controversial whether calcineurin is important for positive or negative selection (Chan et al., 2002; Hayden-Martinez et al., 2000; Kane and Hedrick, 1996).

Receptor proximal signaling elements such as components of the CD3 complex, CD45, Lck, ZAP70, and Vav influence thymic selection. Although other CD3 chains are important for the DN stages of murine thymocyte differentiation (Sommers et al., 2000), CD3δ appears to act at the DP stage (Delgado et al., 2000). γδ T-cells are normal in Cd3δ-deficient mice (Dave et al., 1997), implying that CD3δ is specifically required for αβ DP positive selection. Similarly, a loss of CD45 blocks the transition of DP thymocytes to mature $CD4^+$ and $CD8^+$ SP cells (Byth et al., 1996; Kishihara et al., 1993). Vav1 regulates peptide-specific apoptosis in thymocytes and is required for both positive and negative selection (Kong et al., 1998; Turner et al., 1997). Mice deficient for ZAP70 have DP thymocytes but these cells do not progress to mature $CD4^+$ or $CD8^+$ SP cells and exhibit blocks in both positive and negative selection (Negishi et al., 1995).

The Tec family kinases are also involved in thymic selection. Several members of the Tec family, including Itk and Rlk, are important for setting the signaling thresholds of positive and negative selection (Blomberg et al., 2009; Liao and Littman, 1995; Schaeffer et al., 2000). Id3, a nuclear helix-loop-helix protein regulated by Itk, may play a role in positive selection. (Bain et al., 2001; Rivera et al., 2000).

Morphogens

Wnt signaling results in the activation of many factors required for the growth and survival of DP cells. C-fos, c-jun, Bcl-xL, and various integrins are among the many cell-cycle, survival, and cell adhesion molecules induced by Wnt (Ioannidis et al., 2001; Staal et al., 2004). In addition, Wnt signaling to its downstream target TCF controls CD4 expression in DP cells (Huang et al., 2006).

CD4/CD8 Lineage Commitment

The engagement of MHC class I or II during the positive selection of a DP thymocyte spurs it to extend its survival and differentiate into mature $CD8^+$ or $CD4^+$ SP cells, respectively. This process is called *CD4/CD8 lineage commitment*, and the most current model that provides an excellent framework for the mechanism underpinning CD4/CD8 lineage commitment is called the "kinetic signaling model" (Singer et al., 2008).

Precisely how a DP cell determines that it should become a $CD4^+$ SP or a $CD8^+$ SP cell is beginning to be elucidated (Singer et al., 2008), along with the key transcriptional controllers that are required to induce either lineage choice (Allen, 2009; Bosselut, 2004; Collins et al., 2009). Previous models proposed to account for CD4/CD8 lineage commitment fell into two camps: those based on an instructive mechanism versus those based on a stochastic (random choice) mechanism. The original instructive model proposed that differentiation into a CD4 or a CD8 cell was determined by engagement of the TCR plus CD4 or CD8 by either MHC class II or class I, respectively (von Boehmer, 1986). In other words, thymocytes recognizing MHC class I were instructed to differentiate into $CD8^+$ T-cells, while thymocytes expressing TCRs with affinity for MHC class II were instructed to become $CD4^+$ T-cells. Although initial results using TCR transgenic mice were consistent with this model, later analyses of various mutant mouse strains as well as additional TCR transgenic mice favored a stochastic mechanism (Chan et al., 1993; Davis et al., 1993; Itano et al., 1994; Kaye et al., 1989; Robey et al., 1994; van Meerwijk et al., 1995; von Boehmer, 1996). Subsets of $CD4^+8^{lo}$ and $CD4^{lo}8^+$ T-cells expressing intermediate levels of CD4 and CD8 were identified in different MHC-deficient mutant mouse strains, consistent with the idea that DP thymocytes randomly downregulate the expression of one or the other co-receptor as they move toward becoming mature $CD4^+8^-$ and $CD4^-8^+$ SP cells. This hypothesis was supported by findings that transgenic expression of CD4 in thymocytes could mediate development of $CD4^-8^+$ TCR transgenic cells in class I-deficient mice, and that transgenic expression of CD8 molecules could mediate development of $CD4^+8^-$ TCR Tg cells in class II-deficient mice (Baron et al., 1994; Chan et al., 1994b; Robey et al., 1994; van Meerwijk et al., 1995; von Boehmer, 1996). The stochastic mechanism of CD4/CD8 commitment was also supported by a study examining the deletion of a regulatory motif functioning as a silencer of CD4 expression (Leung et al., 2001). β2M-deficient mice, which usually have only very low numbers of $CD8^+$ T-cells, were transfected with a transgene conferring constant expression of CD4 due to the absence of the silencer. These mice contained mature T-cells, which expressed both CD4 and CD8 and exhibited MHC class II restriction. These cells developed into cytotoxic effectors after antigen stimulation.

The "co-receptor reversal" model, which later gave rise to the "kinetic signaling model," incorporates both stochastic and instructive elements (Brugnera et al., 2000; Sarafova et al., 2005). This model posits that CD8 expression is first downregulated in all DP thymocytes regardless of their MHC

restriction, so that all thymocytes initially become $CD4^+$ cells. These cells then upregulate IL-7R expression. Those thymocytes that subsequently receive both an IL-7 signal and a weak signal delivered by binding to MHC class I resume CD8 expression and downregulate CD4 expression, effectively "reversing" their prior $CD4^+$ phenotype to a $CD8^+$ phenotype (Park et al., 2010). However, those thymocytes that receive a signal through MHC class II maintain their CD4 expression and do not resume CD8 expression. In this case, stromal cells play a prominent role, both as secretors of IL-7 and as suppliers of the pMHC ligand.

Cell-Fate Molecules

Some studies had suggested that Notch-1 may contribute to CD4/CD8 lineage commitment, although its involvement is still the subject of much debate (Deftos and Bevan, 2000; Osborne and Miele, 1999; Robey, 1999; Tanigaki et al., 2004; von Boehmer, 2001). Robey et al. demonstrated that expression of an active form of Notch-1 in developing T-cells resulted in an increase in $CD8^+$ lineage cells and a decrease in $CD4^+$ lineage cells, even if MHC class I was absent (Robey, 1999; Robey et al., 1996). In addition, when Notch-1 signaling was blocked with antibodies, $CD8^+$ SP development was favored (Yasutomo et al., 2000a). Complementary studies revealed that the CD4 silencer that prevents CD4 expression in DN and CD8 SP thymocytes has an HES-1 binding site (Kim and Siu, 1998), and HES genes are known to be regulated by Notch-1 (Bray, 2006). However, other data have suggested that, rather than promoting only the development of $CD8^+$ SP cells, Notch-1 signaling is involved in the maturation of both CD4 and CD8 SP thymocytes (Deftos et al., 1998, 2000). Still others showed that Notch-1 may exert its effect indirectly, by reducing the strength of signal delivered through the TCR (Anderson et al., 2001; Izon et al., 2001). In this hypothesis, cells receiving high Notch-1 signals have reduced signaling through the TCR and consequently adopt the CD8 cell fate, whereas cells receiving lower Notch-1 signals have stronger TCR signals and become CD4 cells. In support of this notion, mice with a loss in Presenilin-1 and -2 function, which are critical for Notch signaling (Bray, 2006), showed a defect in CD4 lineage differentiation from DP cells (Laky and Fowlkes, 2007). However, mice with a conditional deficiency of Notch-1 signaling in thymocytes showed a normal ratio of intrathymic CD4:CD8 T-cells and no differentiation defects (Tanigaki et al., 2004; Wolfer et al., 2002). Thus, further experiments are still required to clarify the issue of how and/or whether Notch regulates CD4/CD8 lineage commitment.

Enzymes and Signaling Molecules

Lck signaling has been proposed as a mechanism of CD4/CD8 commitment because Lck associates with the cytoplasmic tails of both co-receptors (Turner et al., 1990). The model holds that strong Lck signaling helps to favor the differentiation of DP cells into $CD4^+$ SP cells, while little or no signaling results in $CD8^+$ SP cells (Hernandez-Hoyos et al., 2000). The amount of Lck signaling is determined by the MHC restriction of the TCR: MHC class I-restricted TCRs bind to CD8, which is associated with low levels of Lck activity, whereas MHC class II-restricted TCRs bind to CD4, which is associated with about 20-fold higher levels of Lck activity (Salmond et al., 2009). Experiments in which mutated CD8 molecules capable of binding different amounts of Lck were compared showed that the greater the amount of Lck bound to CD8, the more $CD4^+$ T-cells emerged from the DP population (Salmon et al., 1999). Yasutomo et al. devised an in vitro culture system that allowed them to monitor SP development following the binding of a TCR to either wild-type MHC class II (fully capable of binding Lck) or mutated MHC class II lacking the CD4 binding site (decreased Lck binding). In the absence of significant Lck binding, thymocyte differentiation was skewed to $CD8^+$ (Yasutomo et al., 2000b). The authors interpreted their data to mean that the duration of antigen signaling as transduced by Lck determines CD4/CD8 commitment. Additional evidence supporting a commitment model based on quantitative differences in Lck signaling has come out of transgenic studies in which constitutively active or dominant-negative mutants of Lck were used (Hernandez-Hoyos et al., 2000). Overexpression of Lck induced MHC class I-restricted thymocytes to develop into $CD4^+$ T-cells, whereas reduced levels of Lck drove MHC class II-restricted thymocytes down the $CD8^+$ path. Confirmation of these results has been obtained by the introduction of a transgene specifying tetracycline-inducible Lck expression into $Lck^{-/-}$ cells (Zamoyska et al., 2003). Induced expression of Lck resulted in high levels of Lck activity and the exclusive differentiation of $CD4^+$ T-cells. In an interesting twist to the role of Lck in CD4/CD8 lineage commitment, Singer et al. generated a mouse in which CD4, CD8, and class I and class II MHC were deleted (Van Laethem et al., 2007), with the intent to allow the TCR free access to Lck (Haughn et al., 1992)—that is, without Lck being restricted to CD4:MHC-II or CD8:MHC-I requirements for the TCR to have access to its function. In this quadruple knockout mouse, mature T-cells developed, but these were no longer MHC-restricted and appeared to have a CD4-like lineage outcome. This suggests that unrestricted access by TCR to Lck favors a CD4-lineage outcome, and that the normal role of the co-receptors is to ensure that self-MHC restriction is obeyed by tethering Lck availability to MHC recognition by the TCR (Van Laethem et al., 2007).

There is some evidence supporting a role for ERK signaling in CD4/CD8 lineage commitment. In one study, high levels of ERK signaling appeared to favor $CD4^+$ over $CD8^+$ SP development. Transfection of mice with a transgene encoding a constitutively active form of ERK resulted in increased numbers of $CD4^+$ thymocytes but decreased numbers of $CD8^+$ cells (Sharp et al., 1997). Consistent with this result, CD4 maturation was blocked by inhibition of ERK signaling, whereas CD8 development was enhanced (Bommhardt et al., 1999; Sharp and Hedrick, 1999). However, other studies have shown that CD8 differentiation can be blocked by deletion, inhibition, or interference with ERK activity (Alberola-Ila et al., 1995a; Mariathasan et al., 2000; Pages et al., 1999).

Interestingly, mice with a targeted disruption of the interferon regulatory factor 1 (IRF-1) gene have a block specifically

in the development of DP cells into CD8 SP thymocytes as well as a profound reduction in mature $CD8^+$ T-cells (Matsuyama et al., 1993). The development of $CD4^+$ T-cells is normal. IRF1 controls the expression of the *Tap* and other genes required for MHC class I loading, explaining part of the reduction in $CD8^+$ T-cells (White et al., 1996). However, there also appears to be a defect in selection that is intrinsic to $CD8^+$ thymocytes (Penninger et al., 1997).

Chemokines

DP cells that survive positive selection within the cortex and undergo CD4/CD8 lineage commitment become CD4 or CD8 SP cells in the medulla. These thymocytes upregulate their expression of CCR7 so that they can follow chemokine signals (including CCL19 and CCL21) that emanate from mTECs and lead them into the outer medulla (Uehara et al., 2002; Ueno et al., 2002, 2004). It is in this location that negative selection takes place (Fig. 4.3), removing SP thymocytes with autoreactive TCRs from the repertoire and establishing central tolerance (Starr et al., 2003; Takahama, 2006). Accordingly, CCR7-deficient mice exhibit SP cells that cannot migrate properly into the medulla (Ueno et al., 2004). These thymocytes escape negative selection, which is mediated within the medulla, such that autoreactive cells survive and cause the animals to develop autoimmune symptoms (Kurobe et al., 2006).

THE SP STAGE

Negative Thymic Selection

Thymocytes that have been positively selected and undergone CD4/CD8 lineage commitment to become either CD4 or CD8 SP cells migrate into the outer medulla, where they linger for 5 to 7 days (Egerton et al., 1990). Although it was clear by the early 1990s that negative selection of autoreactive thymocytes occurred in the thymus, it took considerably longer to identify the thymic stromal cell types responsible for presenting the selecting ligands and establishing central tolerance (Starr et al., 2003). In 2002, Kyewski et al. demonstrated that mTECs can transiently express a wide range of "housekeeping" and non-thymic tissue-specific proteins, allowing the deletion of reactive T-cell clones before their release to the periphery (Kyewski et al., 2002). mTECs express RNAs encoding tissue-restricted antigens, such as hormones, secreted proteins, membrane proteins, and transcription factors. Experiments in transgenic and nontransgenic mice have confirmed that ectopic expression of a protein by mTECs leads to tolerance of that protein (Anderson et al., 2002; Derbinski et al., 2001; Klein and Kyewski, 2000). Subsequently, it was shown that mTECs synthesize these tissue-restricted self-proteins in such a way that they are taken up by thymic DCs, which then present peptides derived from these antigens on self-MHC to SP thymocytes to induce negative selection (Kyewski and Derbinski, 2004). A TCR that binds tightly to such a pMHC triggers the apoptosis of the SP thymocyte, removing the self-reactive cell from the repertoire (Starr et al., 2003). Other evidence suggests that mTECs may also be involved in the differentiation of regulatory T-cells that can suppress autoimmune responses (Sakaguchi, 2000; Seddon and Mason, 2000).

It is not yet clear whether the expression of tissue-specific genes by mTECs is the result of random derepression of transcription or a more directed activation process. The influence of external factors on mTEC gene expression also remains a mystery. However, a transcription factor called AIRE (autoimmune regulator) appears to play a key controlling role in mTEC-induced central tolerance (Anderson et al., 2002). Both humans and mice deficient for AIRE suffer from autoimmune symptoms that affect multiple organs (Anderson et al., 2002; Bjorses et al., 1998; Ramsey et al., 2002); see also Chapter 31. Analysis of transcription by cells of AIRE-deficient mice showed that mTEC expression of several tissue-specific proteins associated with various autoimmune symptoms was reduced in the absence of AIRE. Furthermore, although the highest levels of AIRE expression occurred in mTECs, AIRE was not required for the differentiation or antigen-presenting function of mTECs. Studies of chimeric animals in which AIRE expression was missing either from hematopoietic cells or from stromal cells showed that peripheral autoimmunity was present only in the latter situation. AIRE is known to interact with the transcriptional co-activator CREB-binding protein (Pitkanen et al., 2000), and it has been suggested that AIRE may participate in a multi-subunit complex in mTECs that activates the transcription of multiple genes encoding tissue-specific proteins. The importance of AIRE for negative selection and tolerance induction in humans is evidenced by the autoimmune symptoms exhibited by patients with autoi mmune-polyendocrinopathy-candidiasis ectodermal dystrophy (APECED, Chapter 21) who have AIRE dysfunction (Aaltonen et al., 1997; Nagamine et al., 1997).

Enzymes and Signaling Molecules

Genes specifically associated with negative selection have been difficult to identify, although many have been implicated (Starr et al., 2003). The engagement of co-stimulatory molecules such as CD28 by binding partners on thymic stromal APCs has been shown to promote negative selection (Amsen and Kruisbeek, 1996; Punt et al., 1997). Interestingly, mice heterozygous for a null Grb2 mutation express subnormal levels of this adaptor and show impaired negative thymocyte selection (Gong et al., 2001). Positive selection is normal in these mutants. Based on the above findings, it has been proposed that negative selection may proceed to ERK activation via Grb2, but that positive selection takes a different route to ERK through RasGRP (Hogquist, 2001). Lck is necessary for the TCR-mediated clonal deletion underlying negative selection (Penninger et al., 1996).

Maturation of SP Cells in the Medulla

The SP thymocytes that emerge from successful positive and negative selection remain in the medulla for another 3 to 5 days, receiving IL-7–mediated survival signals and other stromal cell-derived maturation factors (Zamisch et al., 2005). These thymocytes are not yet functionally mature (Ramsdell et al., 1991), and while some go on to become SP T-cells suitable for

export to peripheral lymphoid tissues, others undergo alternative differentiation and become regulatory T-cells (Hsieh et al., 2006). Both the TCR and CD69 are thought to mediate signaling required for SP maturation, and thymic export may also play a role (Feng et al., 2002; Nakayama et al., 2002). Just prior to export, SP thymocytes in the deep medulla encounter mTECs presenting additional tissue-specific peripheral antigens so that tolerance induction is extended (Kurobe et al., 2006). These mTECs must have experienced signaling mediated by the lymphotoxin-β receptor to achieve the proper degree of differentiation needed to support normal thymic structure and tolerance induction (Boehm et al., 2003). It is after this point that the mature, naïve SP thymocytes leave the thymus at a precise location and via mechanisms that remain to be precisely determined.

CONCLUDING REMARKS

T-cell development is coordinated by the concerted actions of a variety of surface receptors, signal transduction molecules, and transcription factors. Both proper intrathymic localization and specific interactions between thymocytes and thymic epithelial cells and DCs are essential for proper T-cell development. Future challenges include elucidating the precise signals that draw T-cell progenitors into the thymus, orchestrate the coordinated maturation and migration within this organ, and direct the maturation of SP T-cells prior their exit to the periphery. Another task is to continue the refinement of in vitro culture systems for human hematopoietic cells so that human T-cell progenitors can more easily be derived from human HSCs in the future (Awong et al., 2007, 2009). These technologies, plus a greater understanding of T-cell development, will assist in the design of rational therapeutics for the treatment of cancer, autoimmunity, and chronic inflammatory diseases.

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5.

MOLECULAR MECHANISMS GUIDING B-CELL DEVELOPMENT

Antonius G. Rolink and Roxane Tussiwand

INTRODUCTION

B lymphocytes develop from hematopoietic stem cells. In the mouse, B-cell development takes place at different sites in the body during ontogeny, starting at day 8 to 8.5 of gestation in yolk sac and fetal aorta (Cumano et al., 1993; Godin et al., 1993; Palacios et al., 1984). When blood circulation begins at day 9 of gestation, B-cell precursors can still be found in the yolk sac and fetal aorta and, later, around days 10 to 11, in the liver, spleen, and omentum (Ogawa et al., 1988; Owen et al., 1975; Rolink and Melchers, 1991; Solvason and Kearney, 1992). From day 15 to 16 of gestation, B-cell precursors are also found in the bone marrow. After birth, the bone marrow becomes the major site for B lymphopoiesis (Hardy et al., 1991, Osmond, 1991; Rolink et al., 1993, 1994a).

The commitment to various cell lineages, including B-cells, occurs as multipotent hematopoietic stem cells progress through a cascade of several differentiation steps. The classical model of linage determination foresees a hierarchical differentiation, where lineage specification on the one hand progressively defines the commitment and on the other hand restricts other differentiation options.

A complex interplay between specific cytokines and environmental stimuli delivered by stromal cells, together with the gene-expression profile, will determine the developmental cues guiding multipotent progenitors towards a specific fate. B-cell development in mouse and human bone marrow from progenitor (pro) and precursor (pre) B-cells to immature and mature surface immunoglobulin (Ig)-positive B-cells is characterized by changes in:

- Rearrangement of the Ig heavy (IgH) and light (IgL) chain genes
- Expression of intracellular and surface-bound markers
- Cell-cycle status
- In vitro growth properties
- Life expectancy in vivo

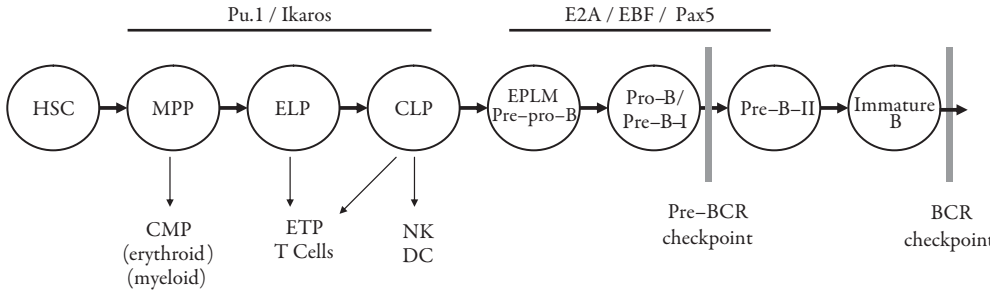
Figure 5.1 summarizes the different stages of B-cell development and the markers expressed at the various stages.

TRANSCRIPTION FACTORS CONTROLLING B-CELL COMMITMENT

Various transcription factors have been shown, by loss- and gain-of-function experiments, to be required for early B-cell development (Matthias and Rolink, 2005). Phenotypic analysis of mice carrying deletions or overexpressing these transcription factors helped to order their requirement during B-cell development. In this section we will focus on the description of transcription factors involved in B-cell commitment.

E2A

The mouse E2A gene encodes for two different proteins of the E-box basic helix-loop-helix (bHLH) family, E12 and E47, which are generated by alternative splicing (Bain et al., 1994;, Zhuang et al., 1994). In B lymphocytes target sequences were found in the IgH and IgL chain enhancer regions (Bain et al., 1994; Zhuang et al., 1994). Despite the broad expression pattern among multipotent progenitors, targeted deletion of E2A leads exclusively to a B-cell deficiency. Almost no $B220^+$ cells are detectable in these mice, with a complete absence of DJ heavy chain rearrangement. Moreover, essential components involved in B-cell development like Rag-1, mb-1, Pax5, and λ5 are absent. In addition, overexpression of E2A in pre-T-cell



	HSC	MPP	ELP	CLP	EPLM/ Pre–pro–B	Pro–B/ Pre–B–I	Large Pre–B–II	Small Pre–B–II	Immaure B
Surface									
FLT3	–	+	+	+	+	–	–	–	–
KIT	+	+	+	+	+	+	–	–	–
CD25	–	–	–	–	–	–	+	+	(+)
IL7R	–	–	–	+	+	+	+	(+)	–
CD43	–	–	–	–	+	+	–	–	–
B220	–	–	–	–	+	+	+	+	+
CD19	–	–	–	–	–	+	+	+	+
Intracellular									
Rag1	–	–	+	+	+	+	–	+	+
Rag2	–	–	+	+	+	+	–	+	+
TdT	–	–	+	+	+	+	–	–	–
D–J (HC)	–	–	–	–	–	+	+	+	+
V–DJ (HC)	–	–	–	–	–	–	+	+	+
V–J (LC)	–	–	–	–	–	–	–	+	+

Figure 5.1 B-cell Development in the bone marrow. Expression of surface and intracellular markers at the different stages of B-cell development.

lines can induce transcription and rearrangement of the IgH chain locus (Schlissel et al., 1991). Collectively, E2A plays a fundamental role in B-cell commitment during early stages of B-cell development when other lineage options are still open.

EBF

A similar phenotype is observed in EBF-deficient mice. The transcriptional activator EBF, also called early B-cell factor, has an early B-cell-restricted tissue expression (Feldhaus et al., 1992; Hagman et al., 1991). Despite the similar phenotype observed in E2A- and EBF-deficient animals, EBF seems to act downstream of E2A, since reintroduction of EBF in E47 null progenitors restores B-cell differentiation (Seet et al., 2004). The suggested model of action for these transcription factors foresees the induction of EBF transcription by E2A, both of which promote Pax5 transcription, and by a feedback loop mechanism, Pax5 is able to reinforce and sustain EBF expression (Northrup and Allman, 2008; Roessler et al., 2007).

PAX5

The transcription factor Pax5, a homeodomain protein, is considered to be the master regulator of B-cell development that controls the commitment to the B-cell lineage by actively repressing the transcription of lineage-inappropriate genes while activating the expression of B-cell lineage-specific genes (Busslinger and Urbanek, 1995; Delogu et al., 2006; Nutt et al., 1999; Schebesta et al., 2007) As for E2A and EBF, also Pax5 deficiency leads to a specific B-cell defect. The block in B-cell development is downstream of the one seen in the absence of E2A and EBF, at the transition from pre-B-I (pro-B) to pre-B-II in the bone marrow. Pre-B-I cells are relatively normal in terms of cell numbers and display DJ heavy chain rearrangement on both alleles, and only proximal V-to-DJ rearrangement can be detected (Fuxa et al., 2004). Surprisingly, the levels of recombination-inducing gene products, such as Rag-1, Rag-2, and TdT, as well as those necessary for the expression of the pre-BCR, like VpreB and λ5, are normal in $Pax5^{-/-}$ pre-B-I cells (Busslinger and Urbanek, 1995). One of the known targets of Pax5 is CD19 (Urbanek et al., 1994), which is undetectable in $Pax5^{-/-}$ pre-B-I cells. However, the absence of CD19 expression cannot explain the $Pax5^{-/-}$ phenotype, since CD19 deficiency has a marginal influence on B-cell development in mice (Engel et al., 1995; Rickert et al., 1995). The defect related to Pax5 deficiency can also not be ascribed to other B-cell lineage-specific genes, since EBF and E2A transcription factors as well as Igα and Igβ B-cell co-receptors are detectable at similar amounts in wild-type and Pax5-deficient pre-B-I cells (Nutt et al., 1997; Schebesta et al., 2002). In contrast to wild-type cells, $Pax5^{-/-}$ pre-B-I cells can, under appropriate in vitro conditions, be driven toward other lineages, namely macrophages, T-cells, natural killer (NK) cells, granulocytes, osteoclasts, and dendritic cells (Busslinger, 2004; Nutt et al., 1999; Rolink et al., 1999b; Schebesta et al., 2002). Pax5-deficient, like wild-type, pre-B-I cells can be maintained indefinitely in vitro on stromal cells in the presence of IL-7 and upon transplantation into lethally irradiated host, can differentiate in vivo into all the above-mentioned lineages (Rolink et al., 1999b; Schaniel et al., 2002a). Moreover, $Pax5^{-/-}$ pre-B-I cells retain extensive self-renewal potential as well as multilineage differentiation capacity, characteristic of hematopoietic stem cells, as shown by serial transplantation experiments (Schaniel et al., 2002b).

The importance of Pax5 in the B-cell lineage is also evident by its maintained expression throughout the B-cell lineage until its downmodulation at the terminal differentiation into plasma cells (Fuxa et al., 2004). There is now broad consensus that Pax5 exerts its action in directing B-cell development by promoting V-to-DJ rearrangement at the heavy chain locus, by inducing expression of B-cell lineage-affiliated genes, and last but not least by repressing a large number of lineage-inappropriate genes (Delogu et al., 2006; Fuxa et al., 2004; Nutt et al., 1997; Pridans et al., 2008; Schebesta et al., 2007).

Recent work of Busslinger et al. introduced the idea that besides commitment, also maintenance of the lineage identity through a specific transcriptional profile is required on immature as well as on fully differentiated cells. They could show that the maintenance of the B-cell lineage is an active process that relies on the transcription factor Pax5. Induced inactivation of Pax5 in committed progenitors is able to induce their differentiation toward other lineages (Schebesta et al., 2007). Moreover, inactivation on mature B-cells induced their dedifferentiation into immature cells; which acquire on one hand self-renewal capacity and on the other hand the potential to differentiate into other lineages (Cobaleda et al., 2007). Gain- and loss-of-function experiments allowed researchers to unravel major mechanisms involved in the development of hematopoietic stem cells into the various lineages. A complex interplay between transcription factors, chemokines, cytokines, and environmental stimuli is needed to progress through development. Moreover, these recent experiments highlighted the possibility that besides a controlled development, also maintenance of a specific lineage identity relies on an active process. While the possibility of lineage conversion remains questionable under physiologic conditions, occurrence of transdifferentiation and dedifferentiation might be critical under diseased conditions.

DEVELOPMENTAL STAGES OF B-CELL DEVELOPMENT

B-cell development can be subdivided into distinct stages from a pro-B and a pre-B-cell to an immature and mature B-cell stage. The rearrangement of the IgH and IgL chain, the expression of intracellular and surface markers, the cell-cycle status, in vitro growth properties, and life expectancy in vivo are all characteristics used to define each stage of B-cell commitment. Figure 5.1 summarizes the early stages of B-cell development with the markers commonly used to identify each step. Upon successful rearrangement of a surface B-cell receptor (BCR), immature B-cells leave the bone marrow and enter the spleen to accomplish the final stages of differentiation into a mature naïve B-cell.

EARLY PROGENITOR OF LYMPHOID-MYELOID CELLS (EPLM) (ALSO CALLED PRE-PRO-B-CELLS)

The search for the physiological counterpart of Pax5-deficient pro-B-cells led to the identification of EPLMs (Balciunaite et al., 2005). EPLMs represent about 0.2 percent of all nucleated bone marrow cells. They are characterized by the expression of B220, CD43, CD135 (FLT3-R), CD127 (IL7R), and CD117 (c-Kit). These cells are contained within the "Fraction A" of Hardy's scheme of mouse B-cell development (Hardy et al., 1991). They are still negative for the B-cell lineage-specific marker CD19 and the IgH and IgL chain loci are still in germline configuration (Rolink et al., 1996). These cells retain various differentiation options as pointed out by the name EPLM, meaning they can give rise in vitro to B, T, and myeloid cells. Upon in vivo transfer, EPLMs were shown to be able to reconstitute the B- and T-cell compartment of immune-deficient mice. Moreover, they were shown to be highly responsive to the cytokine FLT3 ligand. In fact, the number of EPLMs seems to be controlled by the level of this cytokine, where high levels impair B-cell development (Ceredig et al., 2006).

PRE-B-I CELLS (ALSO CALLED PRO-B-CELLS)

EPLMs cells are the direct precursors of pre-B-I cells along the B-cell development pathway. Expression of E2A and EBF induces upregulation of the B-cell master transcription factor Pax5, making the generation of other lineages under physiological conditions rather unlikely. The transcription factor Pax5 induces the surface expression of CD19, the B-cell lineage-specific marker. Therefore, the phenotypic characterization by means of surface markers of pre-B-I cells is identified by the expression of CD117, CD43, and CD19. Besides the surface markers, other elements required for the B-cell lineage commitment start to be expressed at this stage of development: the surrogate light chain (SLC) encoded by the VpreB (gene name: *Vpreb1*, pre-B lymphocyte gene 1) and λ5 genes (gene name: *Igll1*, immunoglobulin lambda-like polypeptide 1) and specific elements of the recombination machinery: Rag-1, Rag-2, and terminal deoxynucleotidyl transferase (TdT) (Grawunder et al., 1995a, 1995b). Moreover, expression of CD135 is at this stage of differentiation lost.

It is at this stage of development that the IgH chain loci undergo their first rearrangement: DH-to-JH rearrangement (ten Boekel et al., 1995). Therefore, mutations affecting the recombination machinery were shown to drastically affect B-cell differentiation at this stage of commitment—that is, severe combined immunodeficiency (SCID) (Bosma et al., 1983), Rag-1 (Mombaerts et al., 1992), and Rag-2 (Shinkai et al., 1992). The same phenotype is also observed in mice that cannot undergo IgH chain rearrangements (J-heavy chain-deficient mice) (Chen et al., 1993).

Pre-B-I cells share with EPLMs the ability to proliferate on stromal cells in the presence of IL-7 (Rolink et al., 1991, 1993; Winkler et al., 1995). Upon in vivo transfer in Rag-2-deficient mice, pre-B-I cells are able to partially reconstitute only the B-cell compartment (Reininger et al., 1992; Rolink et al., 1991, 1994b), indicating that at this stage of development the commitment toward the B-cell lineage has occurred.

In addition to cell intrinsic signals, differentiation, survival, as well as proliferation of B-cell progenitors are regulated by the surrounding environment. The growth factor IL-7 and

its receptor were shown to play a crucial role at this stage of mouse B-cell development. This is best exemplified by a major defect at this early stage of development in mice deficient for the receptor or the cytokine (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). The mechanism by which IL-7 influences B-cell lineage differentiation is probably through increased proliferation and survival.

PRE-B-II

The next developmental stage along the B-cell lineage is characterized by cytoplasmic μ heavy chain expression, which defines the pre-B-II cells (see Fig. 5.1)—that is, they have undergone a productive VDJ rearrangement. Moreover, this cell subset has lost the surface expression of CD117, while most of them gained CD25 expression (Rolink et al., 1994a). Pre-B-II cells can be further subdivided into two different subsets, large and small pre-B-II.

Large pre-B-cells are actively cycling and express on their surface the pre-B-cell receptor (pre-BCR), consisting of the rearranged μ heavy chain in association with the surrogate light chain products (Karasuyama et al., 1996; Rolink et al., 1994a; Winkler et al., 1995). At this stage of development, the recombination machinery is switched off and these cells do not express Rag-1 and Rag-2. Their Ig light chain loci are still in germline configuration and are not yet transcriptionally active (Grawunder et al., 1995a; ten Boekel et al., 1995). It is exactly at this stage of development that allelic exclusion of the second heavy chain allele is likely to be established (Grawunder et al., 1995a).

The importance of this developmental stage, especially the surface expression of the pre-BCR, is best exemplified by mutants where components of the surrogate light chain or the signaling pathway are defective. In mice with a deletion in the transmembrane portion of their μ heavy chain, or deficient for the surrogate light chain components (λ5 or VpreB), large cycling pre-B-II cells are not detectable, resulting in a drastic reduction of small pre-B-cells (Kitamura et al., 1991, 1992; Kitamura and Rajewsky, 1992; Mundt et al., 2001). A similar phenotype is found in mice carrying a deletion in the gene encoding for Igβ (Gong and Nussenzweig, 1996), suggesting that the association between the pre-BCR with Igα and Igβ and thus signaling via the pre-BCR is required for the transition from pre-B-I to pre-B-II cells. In line with this, mice defective for the tyrosine kinase Syk as well as for the adaptor protein BLNK display a block at this early stage of B-cell development, indicating that these are crucial components of the pre-BCR signaling complex (Cheng et al., 1995; Hayashi et al., 2000; Jumaa et al., 1999; Pappu et al., 1999; Turner et al., 1995). The importance of pre-BCR is also evident in experiments in which Rag-1- or Rag-2-deficient mice are supplemented with a transgenic μ heavy chain (Rolink et al., 1994a; Spanopoulou et al., 1994; Young et al., 1994). Rag-1- or Rag-2-deficient mice display a block at the pre-B-I cell stage. However, supplementation with a transgenic rearranged μ heavy chain allows B-cell development to progress up to the small pre-B-II cell stage. Thus, surface expression and signaling via the pre-BCR ensures a proliferative expansion that constitutes a positive selection for pre-B-cells with a productive VDJ rearrangement.

We have shown that this pre-BCR-mediated selection and expansion can be mimicked in vitro. Sorted pre-B-I cells of wild-type mouse cultured in vitro without the addition of cytokines were selected for productive VDJ rearrangement (Rolink et al., 2000). By day 5 of culture, 70 to 80 percent of the recovered cells expressed surface IgM, whereas no expansion and/or selection was observed in pre-B-I cells of λ5-deficient mice. Moreover, single-cell experiments indicated that in wild-type mice approximately 15 percent of pre-B-I cells were able to be selected and expanded. The fact that single ex vivo isolated pre-B-I cells proliferated to different clone sizes in vitro may indicate that the fitness of the different pre-BCRs, by means of pairing ability to the surrogate light chain and the tonic signal delivered, might determine their extent of proliferation and expansion. Moreover, these experiments suggest that the pre-BCR exerts its function in a ligand-independent fashion. Recently it was also shown that pre-BCR signaling downmodulates transcription of the surrogate light chain genes encoding VpreB and λ5 (Parker et al., 2005) and thereby extinguishes its own expression.

The next developmental stage identifies large, cycling pre-B-II cells in which the expression of the surrogate light chain is lost (Karasuyama et al., 1996; Rolink et al., 1994a; Winkler et al., 1995). At this stage of developmenT-cells reactivate the expression of Rag-1 and Rag-2 at the mRNA level but not yet at the protein level (Grawunder et al., 1995a). Moreover, sterile transcripts of the light chain locus become detectable, although it is still in germline configuration (ten Boekel et al., 1995; Yamagami et al., 1999a, 1999b).

When the large cycling pre-B-II cells become resting and small, the recombination machinery is active, the light chain locus is rearranged, and a large proportion of the cells carry rearranged light chain genes (ten Boekel et al., 1995; Yamagami et al., 1999a, 1999b). Experiments by Engel et al. (1995) and Goldmit et al. (2005) suggest that chromatin structural changes rendering the Ig light chain locus accessible for VJ recombination occur earlier within the κ-light chain locus than within the λ-light chain locus. In wild-type mice about half of the Ig-positive B-cells have rearranged only one allele at the κ-light chain locus. Most of these cells show only a single rearrangement, preferentially Jκ1, the most V proximal of the functional J segments. In marked contrast, small pre-B-II cells show a dramatically increased frequency of multiple κ-light chain rearrangements (Yamagami et al., 1999a, 1999b). Moreover, about 20 percent of small pre-B-II cells express a cytoplasmic κ-light chain that is not transferred to the cell surface, although half of the cells have productively rearranged VκJκ segments. This lack of surface BCR expression can be explained by an inefficient pairing of the produced light chain with the already present heavy chain, or the produced BCR might be autoreactive and therefore downregulated. Upon productive light chain rearrangement and efficient pairing with the heavy chain, small pre-B-II cells become surface IgM-positive immature bone marrow B-cells.

IMMATURE B-CELLS

Osmond et al. have determined that an adult mouse produces about 2×10^7 immature B-cells per day (Osmond, 1991, 1993). Immature B-cells are characterized, besides sIgM, by the expression of CD93, recognized by the monoclonal antibodies 493 and AA4.1 (Petrenko et al., 1999; Rolink et al., 1998, 1999a, 2001). At this stage of development B-cells are screened for the expression of an autoreactive BCR. The selection process that occurs will be discussed in detail later. Those immature B-cells that survive this selection process (positive selected ones) will then migrate to the spleen. However, in an adult mouse only about 2×10^6 immature B-cells enter the spleen per day, indicating that 90 percent of them get lost during this transition (Osmond, 1991, 1993; Rolink et al., 1998; Tussiwand et al., 2009). A large part of this loss is likely due to deletion of autoreactive B-cells. Moreover, the quality of the produced BCR and thus its ability to signal also seems to play a crucial role in this transition. This is best exemplified by the fact that Syk-deficient mice and mice expressing a cytoplasmic deleted form of the Igα show a great loss at this stage of B-cell development (Torres et al., 1996; Turner et al., 1995). Moreover, the finding that in CD45-deficient mice more immature B-cells enter the spleen might indicate that the phosphatase CD45 is critically involved in signaling thresholds (Rolink et al., 1999a, 2001).

Also, other mutations were shown to affect the transition of immature B-cells from bone marrow to the spleen. Mice deficient for the transcriptional co-activator OBF show a severe reduction of immature B-cells in the spleen (Schubart et al., 1996). This reduction is even more pronounced in OBF/Oct-2 and OBF/Btk double deficient mice (Schubart et al., 1996, 2001), although molecular mechanisms underlying these defects are still unclear. Overexpression of the anti-apoptotic gene *Bc12* can overcome these defects, which is an indication that survival plays a crucial role in the transition from the bone marrow to the spleen.

TRANSITIONAL B-CELLS

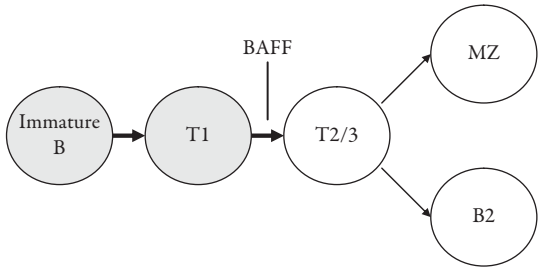
Within the spleen immature B-cells, referred to as transitional B-cells, can be distinguished from their mature counterpart by the expression of CD93 (Rolink et al., 1998). Transitional B-cells can be subdivided into transitional type 1, 2, and 3 (T1, T2, and T3, respectively) according to the expression of CD21, CD23, IgM, and IgD. Figure 5.2 summarizes the different surface markers commonly used to identify the different B-cell subsets present in the spleen. Transitional B-cells are thought to be the direct precursors of the mature naïve B-cells. However, a recent report suggested that T3 cells might represent an anergic B-cell pool (Merrell et al., 2006).

Transitional B-cells are further characterized by their high turnover and their sensitivity to undergo apoptosis upon BCR cross-linking. The latter suggests that also at this stage of development negative selection can still take place (Allman et al., 1992, 1993; Rolink et al., 1998). Some in vivo studies indicated that a significant deletion can occur within the transitional B-cell pool (Allman et al., 1993). On the other hand, turnover studies showed a limited loss of cells along the progression from transitional to naïve mature B-cells. Therefore, the real amount of B-cells that are deleted at this point of differentiation still remains to be determined.

Several mutations affecting this stage of development or the progression to mature B-cells have been identified. Btk-deficient mice have normal numbers of immature splenic B-cells but show a five-fold reduction within the mature compartment. However, the transcriptome of the T1 population is significantly altered in Btk-defective mice (Lindvall et al., 2006). Since the lifespan of mature B-cells in these mice is comparable to wild-type mice, the defect might reflect a reduced transition to the mature compartment (Rolink et al., 1999a). Also in this case increased survival provided by a *Bc12* transgene is able to rescue the phenotype, suggesting that the signaling threshold determines the survival of these transitional B-cell subsets, which is crucial for allowing their differentiation into the pool of long-lived mature B-cells.

The major histocompatibility complex (MHC) class II was also shown to be involved in the transition from immature to mature B-cells in the spleen, but the underlying mechanism is still unclear (Rolink et al., 1999a; Shachar and Flavell, 1996).

The transition from immature to mature B-cells in the spleen is regulated by B-cell activating factor (BAFF) and its receptor. Thus, BAFF- and BAFF-R-deficient mice show a block in the T1-to-T2 B-cell differentiation (Sasaki et al., 2004; Schiemann et al., 2001; Shulga-Morskaya et al., 2004; Yan et al., 2001). BAFF (also termed TALL-1, THANK, BlyS, and zTNF4) is a member of the TNF family (Mackay et al., 2003). It can bind to three different receptors: to BAFF-R, to TACI (transmembrane activator and CAML interactor), and, with lower affinity, to BCMA (B-cell maturation antigen). BAFF-R was shown to be expressed from transitional B-cells up to all mature B-cell subsets, and it is during the transition from T1 to T2 that B-cells become dependent on BAFF–BAFF-R signaling. The binding of BAFF to BAFF-R leads to NF-κB activation mainly through the alternative pathway and ultimately to the upregulation of anti-apoptotic proteins (Sasaki et al., 2006; Tardivel et al., 2004). It is reasonable to assume that interplay between the signaling mediated by the BCR, through the canonical NF-κB pathway, and by BAFF-R through the alternative NF-κB pathway determines the survival and the differentiation of immature B-cell into the mature pool. In vitro studies have shown that immature splenic B-cells treated with BAFF acquire a mature phenotype and become resistant to BCR-cross-linking-induced apoptosis (Batten et al., 2000; Rolink and Melchers, 2002; Rolink et al., 2002). Nevertheless, whether BAFF is necessary for promoting the survival of T2 cells or whether it plays an instructive role during the transition to mature B-cells remains an open question. The defect of BAFF-R deficiency could be rescued by Bcl-2 overexpression, suggesting a survival role for transitional and follicular B-cells (Sasaki et al., 2006; Tardivel et al., 2004). However, since marginal-zone B-cells could not be detected in these mice, an instructive role during marginal-zone B-cell development is likely mediated by BAFF–BAFF-R signaling (Tardivel et al., 2004).



	T1	T2	T3	MZ	B2
IgM	high	high	low	high	low
IgD	low	low	high	low	high
493	+	+	+	–	–
CD21	–	+	+	+	+
CD23	–	+	+	–	+

Figure 5.2 B-cell development in the Spleen. Surface marker expression of different B-cell subsets in the spleen.

MATURE B-CELLS

The mature B-cell compartment can be subdivided into three different subsets: B-1, B-2, and marginal-zone B-cells. Mature B-cells as compared to transitional B-cells in the spleen have lost the expression of CD93, are long-lived, and upon antigenic and mitogenic stimulation respond by proliferation and maturation into Ig-secreting cells (Rolink et al., 1998).

The different mature B-cell compartments are localized within specific microenvironments in the body. Thus, B-1 cells are primarily found in the peritoneal cavity (Hardy and Hayakawa, 2001); B-2 cells are found in the circulation and in the primary follicles of secondary lymphoid organs; while marginal-zone B-cells, as specified by the name, localize in the marginal zone of the spleen, which surrounds the primary follicles (Dammers et al., 1999; Martin et al., 2001).

B-1 cells have a self-renewing potential and are mainly derived from fetal liver stem cells (Hayakawa et al., 2003). The B-1 B-cell subset is the only one that does not require BAFF–BAFF-R signaling for development (Sasaki et al., 2004; Schiemann et al., 2001; Yan et al., 2001). It is responsible for the production of so-called natural antibodies and largely contributes to the T-cell-independent antibody response (Coutinho et al., 1995). The BCR repertoire of B-1 cells was shown to display low affinity for self-antigens, and several studies showed that a low-affinity BCR for self-antigens might drive developing B-cells toward this subset (Hardy and Hayakawa, 2001; Hayakawa et al., 1999).

Marginal-zone B-cells show an activated phenotype as compared to B-2 B-cells; they respond faster and better upon Toll-like receptors triggering (Martin et al., 2001). Moreover, it was shown that marginal-zone B-cells are responsible for the production of the first wave of low-affinity IgM antibodies against bloodborne pathogens (Lopes-Carvalho and Kearney, 2004). Both B-1 and marginal-zone B-cells preferentially respond to T-cell-independent antigens; nevertheless, marginal-zone B-cells can participate in T-cell-dependent antigen responses. As for B-1 cells, the specificity of the BCR might direct developing B-cells into the marginal-zone B-cell lineage (Casola et al., 2004). Moreover, the differentiation of marginal-zone B-cells strictly requires BAFF–BAFF-R signaling, as shown by their complete absence in BAFF- as well as BAFF-R-deficient mice (Sasaki et al., 2004; Tardivel et al., 2004). Besides BAFF, Notch signaling was also shown to be essential for their development; targeted deletion of Notch2 as well as Delta-like-1 results in impaired marginal-zone B-cell compartment without affecting other mature B-cell subsets (Hozumi et al., 2004; Saito et al., 2003). Moreover, Tanigaki et al. (2002) showed that RBP-J, a signaling adaptor protein for all four mammalian Notch receptors, is essential for marginal-zone B-cell development. Conversely, in irradiated Rag-2-deficient mice reconstituted with bone marrow from mice deficient for the negative regulator of Notch signaling MINT, marginal-zone B-cells develop preferentially (Kuroda et al., 2003).

The vast majority of mature B-cells belong to the B-2 B-cell subset. B-2 cells or follicular B-cells are small and resting. They are primarily responsible for T-cell-dependent antibody response and differentiate upon antigenic stimulation into antibody-secreting plasma and memory B-cells. Like B-1 and marginal-zone B-cells, also follicular B-cell development might be influenced by the BCR expressed by the differentiating clone. Several studies showed that mutations causing a weakening of BCR signaling affected the formation of B-1 B-cells but not of B-2 and marginal-zone B-cells (Cariappa and Pillai, 2002; Hardy and Hayakawa, 2001). Moreover, evidence that the expressed BCR might influence the developmental direction toward one or the other B-cell lineage arises from experiments by Rajewsky et al. (Casola et al., 2004), yet direct experimental proof for this is not available.

While on one hand BCR specificity might influence the developmental fate of B-cells, the expression of a signaling competent BCR is required for the maintenance of all three subsets upon maturation (Kraus et al., 2004; Lam et al., 1997). Conditional deletion of the BCR is inducing apoptosis in mature B-cells, suggesting that signaling fitness through tonic BCR signaling is maintaining the pool of mature B-cells. Recent evidence showed that besides playing a crucial role during B-cell development, BAFF–BAFF-R signaling is also required for the maintenance of mature B-cells (Rauch et al., 2009). However, BAFF–BAFF-R signaling plays a crucial role not only in B-cell development and homeostasis but also in B-cell tolerance. It has been shown that mice overexpressing BAFF develop a lupus-like disease (Batten, 2000; Gross, 2000; Khare, 2000; Mackay, 1999). Moreover, elevated BAFF serum levels have been found in patients with autoimmune diseases in which B-cells play an important role (Cheema, 2001; Zhang, 2001). Studies aimed at elucidating the mechanisms by which overexpression of BAFF can result in loss of B-cell tolerance showed that BAFF overexpression does not interfere with the central tolerance program in the bone marrow but rather prevents autoreactive B-cells from being anergized in the periphery (Thien, 2004). Currently, the potential of neutralizing BAFF as a therapy for autoimmunity is being tested both in mouse and clinical settings.

TOLERANCE CHECKPOINTS DURING B-CELL DEVELOPMENT

LARGE PRE-B-II CELLS

At the large-pre-B-II stage of B-cell development, the pre-BCR consisting of the surrogate light chain components, λ5 and VpreB, and the μH chain is expressed. Cells that have undergone a successful V-to-DJ recombination are selected, and the expression of the pre-BCR mediates their proliferative expansion (Bradl and Jack, 2001; Bradl et al., 2003; Ehlich et al., 1994; Gauthier et al., 2002; Melchers et al., 2000; Rolink et al., 2000; ten Boekel et al., 1995; Vettermann et al., 2008). In mice lacking the surrogate light chain components and thus unable to express the pre-BCR, this selection and expansion process fails to operate, resulting in the generation of fewer immature and mature B-cells (Ehlich et al., 1994; Harfst et al., 2005; Melchers et al., 2000; Rolink et al., 2000; Shimizu et al., 2002). Nevertheless, serum antibodies are at normal levels and moreover these mice are able to mount T-cell-dependent and -independent humoral immune responses (Harfst et al., 2005).

Recently it was shown that despite the absence of obvious pathology, surrogate light chain-deficient mice have elevated levels of IgG antinuclear autoantibodies in their serum (Keenan et al., 2008). Thus, autoreactive B-cells can escape negative selection in the absence of the pre-BCR checkpoint. Whether this leakiness in tolerance induction is mediated directly by the pre-BCR, in that pre-B-cells expressing an autoreactive μH chain are deleted at the pre-BCR positive stage of development, or indirectly, by autoreactive B-cells escaping negative selection more readily in situations of reduced B-cell generation, as is the case in pre-BCR-deficient mice, is still open for clarification.

The peripheral mature B-cell compartment in surrogate light chain-deficient mice is about 20 percent the size of that in wild-type mice (Harfst et al., 2005; Shimizu et al., 2002). Moreover, the turnover of mature B-cells in pre-BCR-deficient mice in comparison to wild-type mice is largely reduced (Harfst et al., 2005). Thus, competition for newly formed B-cells to enter the peripheral B-cell pool is far less in surrogate light chain-deficient mice than in wild-type mice. Therefore, it could be envisaged that negative selection of immature autoreactive B-cells in the bone marrow of these mice is less stringent, resulting in higher titers of autoantibodies.

At this point it is worthwhile noting that in humans, as well as in mice, B-cell production in the bone marrow decreases with age and correlates with an increased incidence of autoantibodies in the serum (Dorshkind et al., 2009; Ghia et al., 1996, 2000; Rolink et al., 1993; Yung and Julius, 2008). To test the hypothesis that decreased B-cell production in the bone marrow increases the chance of developing autoimmunity, other mutant mice in which bone marrow B-cell development is reduced should be analyzed.

IMMATURE BONE MARROW B-CELLS

More than 50 years ago Sir Frank Macfarlane Burnet put forward the clonal-selection hypothesis (Burnet, 1959). According to this hypothesis, recognition of foreign antigen by mature B-cells induces clonal expansion and antibody secretion and thus yields an antigen-specific immune response and the induction of memory, whereas newly formed immature B-cells with an autoreactive receptor will die upon encounter with their autoantigen. That death was inevitable for an immature B-cell that had its BCR triggered was challenged about 15 years ago by findings reported by Nemazee et al. (Tiegs et al., 1993) and Weigert et al. (Gay et al., 1993; Radic et al., 1993) and reviewed by Nemazee (2006) and Nemazee and Weigert (2000). The groups of Nemazee and Weigert independently showed that autoreactive immature B-cells, upon encounter of their autoantigen, do not automatically have to die, but can rather eliminate their autoreactive specificity and express a novel non-autoreactive receptor by secondary V(D)J recombination events (Gay et al., 1993; Hertz & Nemazee, 1997; Nemazee, 2006; Nemazee and Weigert, 2000; Radic et al., 1993; Retter & Nemazee, 1998; Tiegs et al., 1993). This process of receptor revision was then called receptor editing (Casellas et al., 2001;Gay et al., 1993; Nemazee and Weigert, 2000; Nemazee, 2006; Radic et al., 1993; Tiegs et al., 1993).

At least three types of immature B-cells can be distinguished: (1) those that express a non-autoreactive receptor with which the IgH and IgL chains pair well and therefore express high levels of the BCR; (2) those that express a non-autoreactive BCR with which the IgH and IgL chains poorly interact and therefore express low levels of the BCR; and (3) those that express an autoreactive receptor. Several studies have now indicated that it is the strength of the BCR signal that determines whether the BCR-triggered cells are allowed to progress in development (positive selection) or will have to undergo receptor editing in order to be rescued (Buhl et al., 2000; Diamant et al., 2005; Fuentes-Panana et al., 2004; Hayashi et al., 2004; Keren et al., 2004; Kouskoff et al., 2000; Pelanda and Torres, 2006; Tze et al., 2005; Verkoczy et al., 2007; Xu and Lam, 2002). Thus, immature B-cells that express a non-autoreactive receptor consisting of an efficiently interacting combination of IgH and IgL chains seem to receive the right amount of BCR signaling (also called tonic or basal BCR signaling), which allows them to progress in development and be positively selected (Buhl et al., 2000; Diamant et al., 2005; Fuentes-Panana et al., 2004; Hayashi et al., 2004; Keren et al., 2004; Kouskoff et al., 2000; Pelanda and Torres, 2006; Tze et al., 2005; Verkoczy et al., 2007; Xu and Lam, 2002). These cells then switch off RAG expression and are allowed to migrate to the spleen. In mutant mice in which this tonic BCR signaling is abrogated and/or impaired, as in CD19-deficient mice, positive selection of immature B-cells is dramatically reduced (Keren et al., 2004).

For the population of immature B-cells that express a non-autoreactive BCR but at low levels, signaling via the receptor is below the threshold required for positive selection, RAG expression is not turned off, and these cells undergo secondary IgL chain rearrangements. If they manage to generate a new IgL chain that interacts well with their IgH chain, resulting in the formation of a non-autoreactive BCR, they will now receive the basal level of BCR signaling required for positive selection.

For the immature B-cells expressing an autoreactive BCR, encounter with their self-antigen generates a strong BCR signal. These cells will therefore not progress in development and will not switch off RAG expression. The latter allows them to undergo secondary IgL chain rearrangements, which can lead to the generation of a new IgL chain. If the resulting new BCR is non-autoreactive and the new IgL chain pairs well with the IgH chain, these cells will be positively selected. Thus, receptor editing can rescue immature B-cells that express a "poor" or autoreactive BCR from apoptosis (clonal deletion); however, the efficiency of this rescue program is still not completely clear. Several studies with transgenic mice have suggested that receptor editing is very efficient in rescuing autoreactive immature B-cells (Ait-Azzouzene et al., 2005; Halverson et al., 2004; Pelanda and Torres, 2006), but whether this conclusion is also warranted for a nontransgenic wild-type situation is unknown. Whether immature B-cells expressing an autoreactive BCR are more easily rescued by receptor editing then those that express a poorly interacting IgH and IgL chain combination is, in our opinion, still unclear.

Yet another important point to be noted is that receptor editing does not automatically result in the deletion of the first expressed IgL chain. A productive secondary rearrangement, taking place either at the unrearranged or nonproductively rearranged allele, will result in the formation of immature B-cells expressing two IgL chains. In fact, it has recently been shown that about half the peripheral mature B-cells derived from immature B-cells that had undergone receptor editing express two IgL chains (Casellas et al., 2007). Moreover, several studies have indicated that dual IgL chain-expressing autoreactive immature B-cells can escape deletion by being positively selected by the non-autoreactive IgL and IgH chain combination (Gerdes and Wabl, 2004; Li et al., 2002, 2005).

TRANSITIONAL SPLENIC B-CELLS

Immature B-cells that are positively selected in the bone marrow will migrate to the spleen and are then called transitional B-cells (Allman et al., 2001; Carsetti et al., 1995; Loder et al., 1999; Rolink et al., 1998, 1999a). Transitional B-cells are still immature and can be distinguished from their mature counterparts by surface marker expression, their short half-life, and their sensitivity to anti-IgM-induced apoptosis—that is, they can still undergo negative selection.

Several in vitro studies have shown that upon anti-IgM triggering, transitional B-cells undergo apoptosis (Allman et al., 2001; Carsetti et al., 1995; Loder et al., 1999; Rolink et al., 1998, 1999a). Moreover, in vivo studies have also indicated that a counterselection against autoreactivity might take place at this stage of development (Meffre and Wardemann, 2008; Wardemann et al., 2003). In fact, some investigators have suggested that only 10 percent of transitional B-cells enter the pool of long-lived mature B-cells (Allman et al., 1992, 1993). However, turnover studies of transitional and mature B-cell subpopulations performed by us suggested very little loss of cells as they progress from transitional to mature B-cells (Rolink et al., 1998). Thus, the transitional stage of B-cell development is yet another checkpoint for BCR autoreactivity. However, how many B-cells are deleted at this developmental stage is still unclear. Furthermore, whether autoreactive transitional B-cells can still be rescued by receptor editing is also unclear.

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6.

SIGNAL TRANSDUCTION BY T- AND B-LYMPHOCYTE ANTIGEN RECEPTORS

Anthony DeFranco and Arthur Weiss

Considerable progress has been made toward our understanding of the processes by which T and B lymphocytes become activated. Antigen-specific activation of these quiescent cells involves the triggering of antigen receptors to induce signal transduction events that can lead to both the cell-cycle progression and differentiation of T and B cells. A remarkable synergy has resulted from a convergence of studies of the most fundamental processes involved in T- and B-cell activation and those involving clinical and experimental immunodeficiency syndromes. Clinical and experimental immunodeficiency states often result when the functions of T- and B-cell antigen receptors (TCRs and BCRs) or critical components in the pathways regulated by these receptors are interrupted or altered.

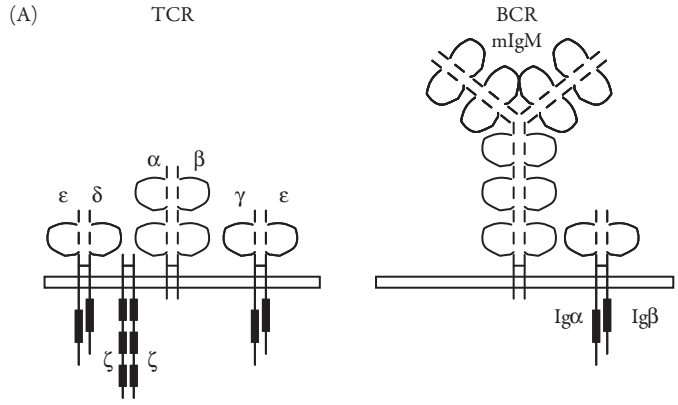
An increasing number of cell surface receptors on T and B cells contribute to the activation of these cells. However, the specificity of the response to antigen dictates that the antigen receptors on these cells play the central role in the initiation of responses. Although the forms of antigens recognized and the effector functions mediated by these two cell lineages are quite distinct, the mechanisms by which the antigen receptors transduce signals and the ensuing molecular events are remarkably similar. Stimulation of the TCR and BCR induces the protein tyrosine phosphorylation of a large number of proteins implicated in signal transduction processes that contribute to cellular activation. Here, we will focus on the means by which the TCR and BCR induce protein tyrosine phosphorylation and the consequences of these phosphorylation events.

ANTIGEN RECEPTORS ON T AND B LYMPHOCYTES

The TCR and BCR are both oligomeric complexes that contain subunits responsible for antigen recognition and other subunits involved in signal transduction (Fig. 6.1A). The repertoires of the clonally distributed ligand-binding subunits of these receptors are entrusted with the responsibility of recognizing the vast array of antigens or peptides bound to major histocompatibility complex (MHC) molecules that may be encountered during interactions with pathogens. In the case of the TCR, the antigen- or ligand-binding subunit consists of productively rearranged $\alpha\beta$ or $\gamma\delta$ heterodimers. The antigen-binding subunit of the BCR consists of the heavy and light chains of membrane immunoglobulin (Ig). Whereas these subunits have all of the information necessary for specific antigen recognition, their short cytoplasmic domains do not contain sufficient information to interact with cytoplasmic molecules involved in signal transduction.

In the case of the TCR, the $\alpha\beta$ or $\gamma\delta$ heterodimers assemble in the endoplasmic reticulum with the invariant CD3 $\varepsilon\gamma$ and $\varepsilon\delta$ chains and a homodimer of the ζ chain (Manolios et al., 1991). The assembly of all of the components is necessary for efficient expression on the plasma membrane. Human or murine T-cell immune deficiency syndromes characterized by lower TCR expression have been associated with CD3γ or ε mutations (Perez-Aciego et al., 1991; Tanaka et al., 1995). Studies in T-cell lines and heterologous cells show that most incomplete complexes are retained in the endoplasmic reticulum or Golgi and shunted toward a degradative pathway (Bonifacino et al., 1989). Unusually placed transmembrane acidic and basic residues appear to play an important role in the interaction of the CD3 and ζ chains with the ligand-binding $\alpha\beta$ or $\gamma\delta$ heterodimers (Call et al., 2002). In addition to being required for proper cell-surface expression, the CD3 and ζ chain dimers are responsible for the signal transduction function of the receptor.

Membrane Ig also assembles with invariant chains involved in signal transduction, called Igα and Igβ. Like the CD3 chains, Igα and Igβ form heterodimers and each chain



(B)

hζ1	N	Q	L	Y	N	E	L	N	L	G	R	R	E	E	–	Y	D	V	L
hζ2	E	G	L	Y	N	E	L	Q	K	D	K	M	A	E	A	Y	S	E	I
hζ3	D	G	L	Y	Q	G	L	S	T	A	T	K	D	T	–	Y	D	A	L
hCD3γ	D	Q	L	Y	Q	P	L	K	D	R	E	D	D	Q	–	Y	S	H	L
hCD3ε	N	P	D	Y	E	P	I	R	K	G	Q	R	D	L	–	Y	S	G	L
hCD3δ	D	Q	V	Y	Q	P	L	R	D	R	D	D	A	Q	–	Y	S	H	L
hDAP12	E	S	P	Y	Q	E	L	Q	G	Q	R	S	D	V	–	Y	S	D	L
rIgE FcRγ	D	A	V	Y	T	G	L	N	T	R	N	Q	E	T	–	Y	E	T	L
rIgE FcRβ	D	R	L	Y	E	E	L	–	H	V	Y	S	P	I	–	Y	S	A	L
mIgα	E	N	L	Y	E	G	L	N	L	D	D	C	S	M	–	Y	E	D	I
mIgβ	D	H	T	Y	E	G	L	N	I	D	Q	T	A	T	–	Y	E	D	I
BLV gp30	D	S	D	Y	Q	A	L	L	P	S	A	P	E	I	–	Y	S	H	L
EBV LMP-2	H	S	D	Y	P	L	L	G	T	Q	D	Q	S	L	–	Y	L	G	L
SIV Nef	G	D	L	Y	E	R	L	L	R	A	R	G	E	T	–	Y	G	R	L
KSHV	L	Q	D	Y	Y	S	L	H	D	L	C	T	E	D	–	Y	T	Q	P
Consensus	–	–	–	Y	–	–	L	–	–	–	–	–	–	–	–	Y	–	–	L

Figure 6.1 *ITAMs and antigen receptors.* (A) Schematic representation of the T-cell antigen receptor (TCR) and B-cell antigen receptor (BCR) structure. (B) Immunoreceptor tyrosine-based activation motifs present in hematopoietic receptors involved in antigen recognition and in viruses that infect T or B cells. h, human; r, rat; m, mouse; BLV, bovine leukemia virus; EBV, Epstein-Barr virus; SIV, simian immunodeficiency virus; KSHV, Kaposi's sarcoma–associated herpes virus.

has a single extracellular Ig-like domain and intracellular domains with signaling function. Recent data suggest that a single Igα/Igβ dimer is associated with a dimer of membrane Ig (Schamel and Reth, 2000; Sohn et al., 2006). As is the case with the TCR, efficient cell-surface expression of membrane Ig requires association with Igα and Igβ. Although charged amino acid residues are not present in the transmembrane domains of BCR subunits, these domains do interact with each other, and this interaction is necessary for assembly (Venkitaraman et al., 1991). Thus, the BCR and TCR are both obligate multi-subunit complexes in which antigen-binding functions and signal transduction functions are provided by distinct chains.

In addition to the antigen receptors found on mature lymphocytes, related receptors are found on pre-B cells in the bone marrow and on immature double negative ($CD4^-/CD8^-$) thymocytes (Roth and DeFranco, 1995; von Boehmer, 2005). These receptors contain only one of the two antigen-binding chains, the one that is generated by recombination first during development of the cell. The place of the other antigen-binding chain is taken by a surrogate for the second chain. In B cells, this "pre-BCR" contains the Ig μ heavy chain together with the λ_5 and V_{preB} proteins, each of which mimics one half of the Ig light chain of the BCR (Bankovich et al., 2007). This pre-BCR also contains Igα/Igβ heterodimers. Similarly, the pre-TCR contains a complex of the TCRβ chain with an α chain surrogate called pre-Tα, and this complex is associated with CD3 and ζ chains. There is some evidence, in the case of both of these immature receptors, that they dimerize or oligomerize to induce signaling in a ligand-independent manner (Bankovich et al., 2007; Irving et al., 1998; Melchers, 2005; Yamasaki et al., 2006). In any case, these receptors play critical roles in allowing developing lymphocytes to sense the successful rearrangement of the first antigen receptor gene and thereby promote passage through a developmental checkpoint and initiate a limited proliferative expansion, after which rearrangement at that locus ceases and rearrangement of the Ig light chain gene or the TCRα gene increases. For this reason, genetic defects in TCR or BCR signaling reactions result in an early block in lymphocyte development, a prime example being the strong block in B-cell development in X-linked agammaglobulinemia caused by mutations in the gene encoding Btk (Conley et al., 2000; Lindvall et al., 2005).

The CD3 and ζ chains of the TCR and the Igα and Igβ chains of the BCR have substantial cytoplasmic domains, which contain sequences responsible for the signal transduction function of these receptors. These signal transduction functions were initially definitively established through the use of chimeric receptors in which the cytoplasmic domains of several of these proteins were linked to the extracellular and transmembrane domains of heterologous proteins (Irving and Weiss, 1991; Law et al., 1993; Romeo and Seed, 1991). Although these cytoplasmic domains do not encode enzymatic functions, they can confer TCR and BCR signal transduction functions to heterologous receptors if expressed in hematopoietic cells having the appropriate downstream signaling molecules. This signal transduction function is encoded in a sequence motif termed ITAM (for immunoreceptor tyrosine-based activation motif), which is present as a single copy in all of the CD3 chains and the Igα and Igβ chains, and as three copies in the ζ chain (Fig. 6.1A and B) (Flaswinkel et al., 1995). ITAMs are also present and functionally active in the cytoplasmic domains of the non-ligand-binding subunits of the high-affinity IgE Fc receptor (β and Fcγ chains), the phagocytic Fcγ receptors on macrophages, and the DAP-12 chain that associates with natural killer (NK) cell-activating receptors. Interestingly, the ITAM sequence motif, D/ExxYxxL(x)6–8YxxL (one letter amino acid code; x = any amino acid), in many of these chains is encoded by two exons with similar organization, suggesting a common evolutionary origin (Wegener et al., 1992). Indeed, all of these receptors involved in antigen recognition use similar mechanisms to transduce signals. Mutagenesis studies have shown that both tyrosines and both leucines are critical for the ability of ITAMs to confer signal transduction function to heterologous

receptors (Sefton and Taddie, 1994). The amino acid residues surrounding the conserved tyrosines and leucines can be quite variable. A major question that arises and remains unsettled is whether ITAMs, which contain such variable sequences, have equivalent functions or whether part of their functions are distinctive. Some investigations have suggested that multiple ITAMs are present in TCRs and BCRs to recruit distinct signaling molecules (Clark et al., 1992; Frank et al., 1990; Siemasko and Clark, 2001). Others have suggested that the presence of multiple ITAMs may play a role in signal amplification (Irving et al., 1993; Law et al., 1993; Love and Shores, 2000). In mouse experiments involving genetic deletion of all or part of the CD3ζ ITAMs, it was shown that the CD3γ, δ, ε subunits are sufficient for TCR signaling events and effector functions and that the CD3ζ ITAMs do not play any exclusive role (Ardouin et al., 1999). However, experiments with receptors containing multimers of ITAMs suggest signal amplification may be a role for the 10 ITAMs of the TCR (Irving et al., 1993; Love and Shores, 2000). Moreover, sufficient numbers of ITAMs and their signaling function may prevent the development of autoimmunity (Holst et al., 2008).

B-cell development requires at least one functional ITAM in the Igα/Igβ heterodimer, but defects are seen in mice with mutations in either ITAM. Mice containing mutations of the ITAM tyrosines in Igα did not exhibit any major defects in the development of follicular B cells, whereas there was a reduction in B-1 and marginal-zone B cells. Combining the Igα mutation with a truncation in Igβ resulted in a marked arrest in B-cell development at the pro-B cell stage (Kraus et al., 2001; Reichlin et al., 2001), demonstrating the requirement for functional ITAMs on at least one of the signal transducing chains of the BCR for progression through early development. Mice carrying a truncation in the cytoplasmic tail of Igβ alone progress to the immature stage and respond normally to BCR ligation; however, they are arrested at this stage in the bone marrow and die by apoptosis. These observations indicate that Igα and Igβ might have distinct biological activities in vivo in addition to some redundancy.

As in other situations where pathogens have usurped or targeted normal cellular machinery to their benefit, at least four viruses that infect T or B cells encode ITAMs that appear to play an important role in their pathogenesis. The bovine leukemia virus, which can transform B cells, contains an ITAM in the cytoplasmic domain of the gp30 envelope glycoprotein that is critically important for viral infection and/or replication (Willems et al., 1995). An ITAM is also present in the latent membrane protein 2 (LMP2) of the Epstein-Barr virus (EBV) and has been implicated in maintaining a state of viral latency in transformed B-cell lines (Fruehling and Longnecker, 1997). LMP2 serves to recruit E3 protein ubiquitin ligases that target BCR-activated protein kinases, thereby downregulating BCR signaling, but also induces a low level of constitutive ITAM signaling (Pang et al., 2009). A rare mutation of the Nef protein of the simian immunodeficiency virus results in the creation of an ITAM sequence (Du et al., 1995). This mutation is associated with the unusual ability of this viral isolate to infect and replicate in primary resting T cells and cause a fulminant viral infection. Finally, human herpesvirus 8 (HHV8; also known as Kaposi's sarcoma [KS]-associated herpesvirus, or KSHV), which causes Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease, encodes the K1 gene product that contains an ITAM-like sequence in its C-terminal cytoplasmic tail. Expression of this protein leads to ligand-independent signaling in B cells that is abrogated upon mutation of the ITAMs (Lagunoff et al., 1999). Thus, the ITAM plays a critical role in normal T- and B-cell antigen receptor function, and some pathogens have capitalized on this function.

ITAMS OF T- AND B-CELL ANTIGEN RECEPTORS INTERACT WITH DISTINCT FAMILIES OF PROTEIN TYROSINE KINASES

Stimulation of the TCR and BCR leads to the activation of protein tyrosine kinases (PTKs) that are critical for lymphocyte responses to antigen. Two families of cytoplasmic PTKs, members of the Src and Syk/ZAP-70 families, have been implicated in the most proximal signaling events induced by these receptors. Indeed, considerable evidence has accumulated to suggest a model in which the TCR and BCR ITAMs interact with these two families of PTKs in a sequential and coordinated manner (Fig. 6.2) (Weiss, 1993). Whereas the members of the families of the PTKs that are expressed in T and B cells differ, they appear to subserve similar functions.

In B cells and in T cells, the earliest event associated with antigen receptor stimulation is the tyrosine phosphorylation of ITAMs (Iwashima et al., 1994; Law et al., 1993). In some cases, in ex vivo T cells, phosphorylation of the ITAM tyrosines is present in the basal state (van Oers, Tao et al., 1993). Both the inducible and basal phosphorylation of ITAM tyrosines depends on members of the Src family (Iwashima et al., 1994; Richards et al., 1996; Saijo et al., 2003; van Oers, Killeen et al., 1996). Src family kinases expressed in T cells are Lck and Fyn and those expressed in B cells are primarily Lyn, Fyn, and Blk. These kinases are peripheral membrane proteins characterized by the following: (1) a unique N-terminal domain that is myristylated in all family members and also may be palmitoylated; (2) an SH3 domain involved in mediating protein–protein interactions by binding to proline-rich sequences; (3) an SH2 domain involved in mediating protein–protein interactions by binding phosphorylated tyrosine residues in the context of particular neighboring amino acid residues; (4) a catalytic domain; and (5) a carboxy-terminal domain that contains a negative regulatory site of tyrosine phosphorylation.

In T cells, the function of Lck is especially critical for TCR signal transduction (Straus and Weiss, 1992; van Oers, Killeen et al., 1996), although Fyn is thought to play roles in some circumstances (Filby et al., 2007; Mamchak et al., 2008). Lck and Fyn both co-immunoprecipitate with the TCR complex and can interact with ITAMs (Beyers et al., 1992; Samelson et al., 1990), but genetic studies with cell lines and mice have indicated a more important role for Lck in the phosphorylation of the ITAMs (Straus and Weiss, 1992; van Oers, Killeen et al., 1996). However, in the absence of Lck, or when Lck function

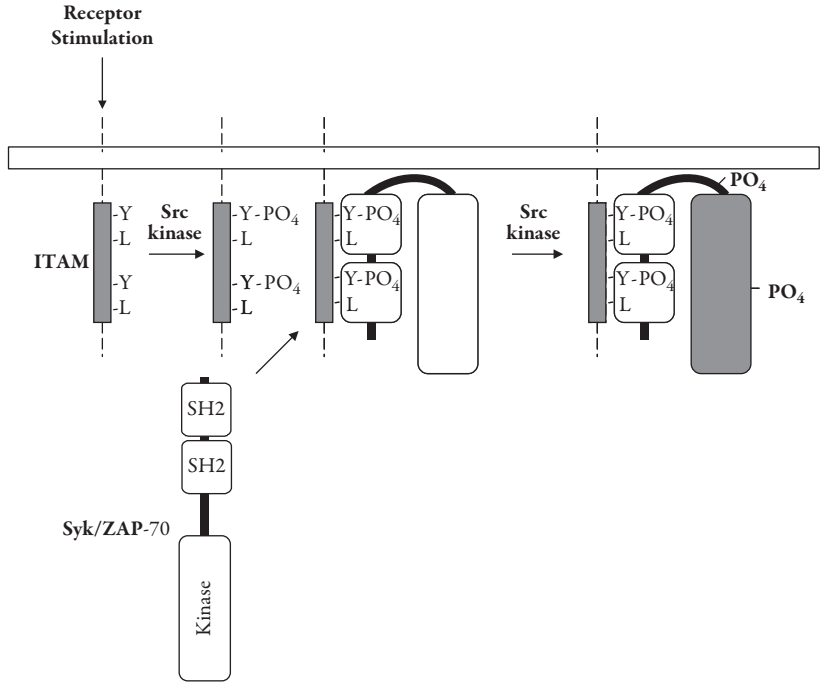


Figure 6.2 *Model for sequential interaction of Src and Syk/ZAP-70 protein tyrosine kinases with an ITAM*. See text for details. The subsequent interaction of the Src family kinase, involving its SH2 domain, with the phosphorylated Syk or ZAP-70 kinase is not depicted.

might be limiting, Fyn may be able to play a compensatory role. This view is consistent with the developmental phenotypes seen in mice deficient in one or both of these kinases: mice deficient in Fyn have no developmental abnormality of conventional αβ T cells but they lack NK1. 1+ T (NKT) cells (Appleby et al., 1992; Eberl et al., 1999; Stein et al., 1992), whereas the loss of Lck is associated with a severe but incomplete arrest in T-cell development (Molina et al., 1992). The residual development in the latter case is completely absent in thymocytes from mice deficient in both kinases (van Oers, Lowin-Kropf et al., 1996). These thymocytes exhibit a complete developmental block at a stage where the function of the pre-TCR is important (Fehling et al., 1995). Presumably, this reflects the inability of the pre-TCR to transduce signals in the absence of Lck or Fyn.

A unique feature of Lck is the ability of its N-terminal region to associate with the cytoplasmic tails of the CD4 and CD8 co-receptors, which are involved in recognition of MHC class II or I molecules, respectively (Chow and Veillette, 1995). A primary role for Lck function in the initiation of TCR signaling is also consistent with its association with CD4 and CD8. The co-localization of CD4 or CD8 with the TCR during peptide antigen-MHC molecule recognition may serve to localize Lck in close proximity with the TCR, facilitating its participation in early signaling events.

In B cells the Src family kinases are also believed to be responsible for ITAM phosphorylation, although Syk may contribute as well. For example, in a chicken B-cell line, DT-40, Lyn is apparently the only Src family kinase expressed. Disruption of the Lyn gene in these cells decreased anti-IgM-induced tyrosine phosphorylation of Igα and Igβ but did not eliminate it (Takata et al., 1994). Moreover, subsequent signaling events were decreased but not eliminated. Conversely, when the BCR was expressed in the AtT-20 rat pituitary cell line following the introduction of cDNA expression vectors, BCR stimulation led to strong tyrosine phosphorylation of Igα and Igβ but minimal downstream signaling. These cells express Fyn but not Syk, Lyn, or Blk. Introduction of Syk restored some downstream signaling events and also boosted the stimulation-dependent phosphorylation of Igα and Igβ (Richards et al., 1996). In murine splenic B cells, Lyn is the most abundant Src family member present, although substantial amounts of Blk and Fyn are also detectable. B cells from mice rendered deficient in Lyn by targeted gene disruption exhibit significantly delayed tyrosine phosphorylation of Igα after BCR stimulation (Chan et al., 1997; Nishizumi et al., 1995), indicating that Lyn is important but not essential for Igα phosphorylation. Mice genetically defective in Lyn, Blk, and Fyn, the three major Src family kinases in B cells, exhibit a strong block at the pre-BCR signaling checkpoint, whereas the single and double mutant mice have a less severe defect, strongly suggesting that these three kinases all contribute significantly to pre-BCR signaling (Saijo et al., 2003). Thus, the situation is a bit more complex than in T cells, but again, Src family kinases are primarily responsible for phosphorylation of Igα and Igβ ITAM tyrosines.

In the absence of co-receptor involvement, the low stoichiometry of the documented interactions of the Src family members with the TCR and BCR, as well as the apparent redundancy in some functions among these PTKs, raises questions about how TCR and BCR stimulation induces these kinases to phosphorylate the ITAMs. Receptor ligation does appear to increase the catalytic activity of these PTKs (Saouaf et al., 1994). Some recent studies suggest that

a conformational change occurs following TCR stimulation. This can be detected in at least some studies as increased accessibility of a proline-rich motif in the cytoplasmic domain of CD3ε to binding by the N-terminal SH3-domain of the adaptor protein, Nck, or to a monoclonal antibody directed against the proline-rich motif (Gil et al., 2002; Martinez-Martin et al., 2009). The inability of some to document the importance of the proline-rich motif in initiating signaling raises questions regarding the significance of the conformational change (Mingueneau et al., 2008). However, it has been suggested that the motif may help couple the TCR to Lck, directly or indirectly. Recent total internal reflection fluorescence microscopy (TIRF) studies examining BCR signaling in response to a monomeric antigen presented in a planar lipid bilayer have provided evidence that conformational changes may play a role in BCR signaling in such situations. These studies indicated that antigen binding induces conformation changes in the constant domain of mIg closest to the membrane, which then promotes BCR oligomerization and signaling (Tolar et al., 2009). Alternatively, or additionally, engagement of antigen receptors may promote interactions with Src family kinases by altering their location within the plasma membrane from the non-membrane raft region to the membrane raft region, which is highly enriched in Src family kinases (see below).

The stoichiometry of ITAM phosphorylation is low, even in systems where receptor stimulation is maximal. Some studies suggest that some of the tyrosines of TCRζ or CD3 chains may be sequestered in the unphosphorylated state by the hydrophobic environment of the plasma membrane (Aivazian and Stern, 2000; Xu et al., 2008). In any case, the association of the antigen receptor and Src family kinase could be based on an interaction between the SH2 domain of the Src family PTK and a small percentage of ITAMs that may be tyrosine phosphorylated in the basal state. The recruitment of Src family PTKs to phosphorylated ITAMs is attractive in view of studies suggesting that the tyrosine phosphorylated YEEI sequence, similar to the YxxL sequences in ITAMs, is a preferred binding site for the SH2 domains of Src kinases (Songyang et al., 1993). Most ITAMs have at least one negatively charged amino acid residue between the tyrosines and leucines (Fig. 6.1B). Indeed, cross-linking of chimeric transmembrane proteins with Igα or Igβ ITAMs leads to induced association of Lyn (Law et al., 1993). Moreover, the inducible phosphorylation of ζ chain ITAMs requires the function of the Lck SH2 domain (Straus et al., 1996). This could reflect a positive feedback loop whereby once a few ITAM tyrosines are phosphorylated, Lck binds and then phosphorylates other ITAMs of neighboring receptors aggregated by the triggering stimulus.

Both tyrosines of an ITAM are necessary for its signal transduction function (Sefton and Taddie, 1994). This dependency on both tyrosines likely reflects the requirement for tyrosine phosphorylation of both sites for high-affinity binding of a second type of PTK, ZAP-70 and/or Syk. ZAP-70 and Syk have a similar overall structure, containing N-terminal tandem SH2 domains and a C-terminal catalytic domain. ZAP-70 is expressed predominantly in T cells and NK cells, whereas Syk is more broadly expressed within the hematopoietic lineages. ZAP-70 and Syk bind to doubly phosphorylated ITAMs with relatively high affinity via an interaction that depends on both of their SH2 domains (Bu et al., 1995; Iwashima et al., 1994; Wange et al., 1993). The crystal structure of the ZAP-70 tandem SH2 domains bound to a doubly phosphorylated TCRζ ITAM peptide confirms that both SH2 domains interact with a single, doubly phosphorylated ITAM and helps explain their cooperative binding (Hatada et al., 1995). Recent mutagenesis and structural data suggest that ZAP-70 and probably Syk are in an inactive autoinhibited conformation in the unbound state in which the SH2 domains are not optimally aligned for binding to a doubly phosphorylated ITAM (Brdicka et al., 2005; Deindl et al., 2007). ITAM binding stabilizes alignment of the SH2 domains and transmits a conformational change that can contribute to kinase domain activation.

If ITAMs are phosphorylated in the basal state, as has been observed in ex vivo thymocytes and peripheral T cells, ZAP-70 and/or Syk are then bound and poised to respond to receptor stimulation (van Oers, Killeen et al., 1994). Thus, the induction of PTK activity following Syk/ZAP-70 recruitment to the receptor complexes likely involves additional interactions between the Src and Syk/ZAP-70 PTKs. In vitro studies suggest that the binding of Syk, but not ZAP-70, to ITAMs can lead to its activation (Shiue et al., 1995). However, simple binding of Syk and ZAP-70 to ITAMs in vivo is probably not sufficient for stimulation of their kinase activity and subsequent signal transduction, since Syk and ZAP-70 bound to the ITAMs in ex vivo thymocytes are not activated (van Oers, Killeen et al., 1994). Rather, both Syk and ZAP-70 kinase activities are regulated by phosphorylation of tyrosine residues within a region between the SH2 domains and kinase domain (interdomain B) as well as tyrosine residues within the activation loop of their catalytic domains (Brdicka et al., 2005; Chan et al., 1995; Wange et al., 1995). In the case of ZAP-70, phosphorylation of tyrosines in interdomain B stabilizes the active conformation of ZAP-70 (Brdicka et al., 2005; Deindl et al., 2007). The phosphorylation of the tyrosines in interdomain B can be mediated by Lck or Fyn but not by ZAP-70 itself (Brdicka et al., 2005). In contrast, Syk can activate itself, and this is seen in overexpression studies in Cos cells or upon cross-linking of a chimeric protein in which Syk has been fused to the cytoplasmic domain of a transmembrane protein (Couture et al., 1994; Kolanus et al., 1993). However, Syk is probably more efficiently activated by Src family PTKs such as Lyn than by itself. Thus, the Src kinases can play a critical role in Syk and ZAP-70 catalytic activation by phosphorylating regulatory tyrosines. This is then likely followed by trans-autophosphorylation of tyrosines in their activation loop, which stabilizes the active conformation of the catalytic domain.

In addition to the phosphorylations that lead to activation of ZAP-70 and Syk kinase activity, there are a number of tyrosines in these proteins that become tyrosine phosphorylated, which leads to the recruitment of signaling components. Tyrosine phosphorylation of Y292 in ZAP-70 leads to negative regulation (Kong et al., 1996; Magnan et al., 2001; Zhao and Weiss, 1996). Tyrosines in interdomain B, Y315 and Y319, that are involved in autoinhibition in the basal

unphosphorylated state also have positive regulatory functions following their phosphorylation (Gong et al., 2001; Magnan et al., 2001). These latter phosphorylation sites are involved in recruitment of substrates that contain SH2 domains with specificity for the phosphorylated sequence motifs, in a manner analogous to that observed with PTK growth factor receptors. Proteins that have been reported to bind to either Syk or ZAP-70 include Lck (Straus et al., 1996; Thome et al., 1995), Lyn (Sidorenko et al., 1995), Shc (Katzav et al., 1994; Nagai et al., 1995), PLCγ1 (Law et al., 1996), SHIP (a phosphoinositide 5 phosphatase) (Crowley et al., 1996), and Cbl (Fournel et al., 1996). The functions of some of these signaling components will be discussed in a later section. The interaction of one of these sites with Src kinases is of particular interest. A stable complex of Lck and ZAP-70 or Syk has been observed following TCR stimulation (Straus et al., 1996; Thome et al., 1995). The interaction appears to depend on the binding of the Lck SH2 domain to tyrosine phosphorylated residues in the interdomain B region of ZAP-70 (Y319) or Syk (Y346). This may serve not only to stabilize the active conformation of ZAP-70 or Syk, but also to stabilize and potentiate the activity of the bound Src kinase.

The relative contribution of the two families of PTKs toward phosphorylation of downstream signaling substrates has not been entirely clarified. However, activation of Lck alone by cross-linking CD4 or CD8 fails to mimic TCR stimulation. This suggests a critical function provided by Syk and ZAP-70 in later signal transduction events. Successful reconstitution of Syk-deficient B cells or ZAP-70-deficient T cells with ZAP-70 or Syk depends on the catalytic functions of these PTKs and the presence of the activating tyrosine of the kinase domain (Y518, Y519 for Syk, and Y493 for ZAP-70) (Kong et al., 1996; Takata and Kurosaki, 1995; Williams et al., 1998). Thus, a contribution of both Src and Syk/ZAP-70 catalytic function is important for effective downstream signaling events.

Both ZAP-70 and Syk play critical roles in lymphocyte development, presumably because signal transduction through immature and mature antigen receptors is critical for transition through certain developmental checkpoints. Mice and humans deficient in ZAP-70 exhibit distinct developmental arrests and marked impairment in TCR signal transduction function (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995); see Chapter 14. In Syk-deficient mice, profound B-cell developmental arrest and abnormal development of the vascular and lymphatic vessels leading to shunts in blood flow that contribute to high-output cardiac failure are observed (Cheng et al., 1995; Turner et al., 1995). The B-cell developmental arrest in these mice occurs at the same stage as seen with mutations that prevent expression of the membrane-bound form of the Igμ heavy chain. Thus, it is thought that the developmental block results from impaired pre-BCR function. This interpretation is consistent with the impaired BCR signal transduction function observed in an avian B-cell line deficient in Syk (Takata et al., 1994). Thus, analysis of loss-of-function mutations of Syk or ZAP-70 supports the conclusion that these PTKs play critically important roles in TCR and BCR signal transduction function.

In some systems it has been suggested that Syk and ZAP-70 may be interchangable (Gelfand et al., 1995; Kong et al., 1995). One clear difference is that Syk appears to be less dependent on interactions with Src kinases than is ZAP-70 (Chu et al., 1996; Kolanus et al., 1993). Syk and ZAP-70 are probably not entirely redundant during early thymocyte development. In competitive repopulation studies, Syk appears to play a more important function earlier, one that is critical for initial pre-TCR signaling (Palacios and Weiss, 2007). A pre-TCR Syk-dependent signal induces expression of ZAP-70, which then plays a preferred role in clonal expansion past the pre-TCR checkpoint and in subsequent T lineage stages of development and in mature T-cell function. There is other evidence for distinct functions. For instance, the αβ T-cell lineage is primarily affected in mice made deficient in ZAP-70 (Negishi et al., 1995), whereas the γδ lineage of T cells in epithelial tissues seems to be defective in Syk-deficient mice (Mallick-Wood et al., 1996). Thus, many of the functions of these PTKs overlap, but they appear to have unique functions as well.

REGULATION OF ANTIGEN RECEPTOR SIGNAL TRANSDUCTION

CO-RECEPTORS ON T CELLS

The immune system has evolved great sensitivity to antigen to allow immune responses to be initiated early upon infection with pathogens. While receptors with high affinity exist within the derived repertoire of antigen receptors on T and B cells, the immune sytem has also developed mechanisms to increase the sensitivity of responsiveness until the clonal expansion of cells with high-affinity receptors can take place. T and B cells both have distinct co-receptor molecules that function in concert with antigen receptors to recognize antigen and induce signaling events. Here we will restrict our use of the term *co-receptors* to refer to molecules that along with the antigen receptor recognize intact antigen or the antigenic peptide antigen–MHC complex.

The positive selection of most αβ thymocytes and the primary response of αβ T cells to antigen usually depend on the functional contributions of the co-receptors CD4 and CD8. The CD4 and CD8 molecules are integral membrane glycoproteins whose extracellular domains bind to the β2 domain of class II MHC molecules or the α3 domain of class I MHC molecules, respectively, on antigen-presenting cells (Rudolph et al., 2006). These interactions are of relatively low affinity and may depend on the simultaneous binding of the TCR as well. In fact, some evidence suggests that a TCR-initiated signaling event may be required for the recruitment and effective participation of the CD4 coreceptor (Xu and Littman, 1993). An alternative but not mutually exclusive view is that the CD4 or CD8 participation stabilizes the low-affinity TCR-MHC/peptide interaction and also potentiates TCR signal transduction by delivering Lck to the TCR complex (Fig. 6.3).

The cytoplasmic domains of CD4 and CD8 bind to the unique N-terminal region of Lck through a cysteine-containing

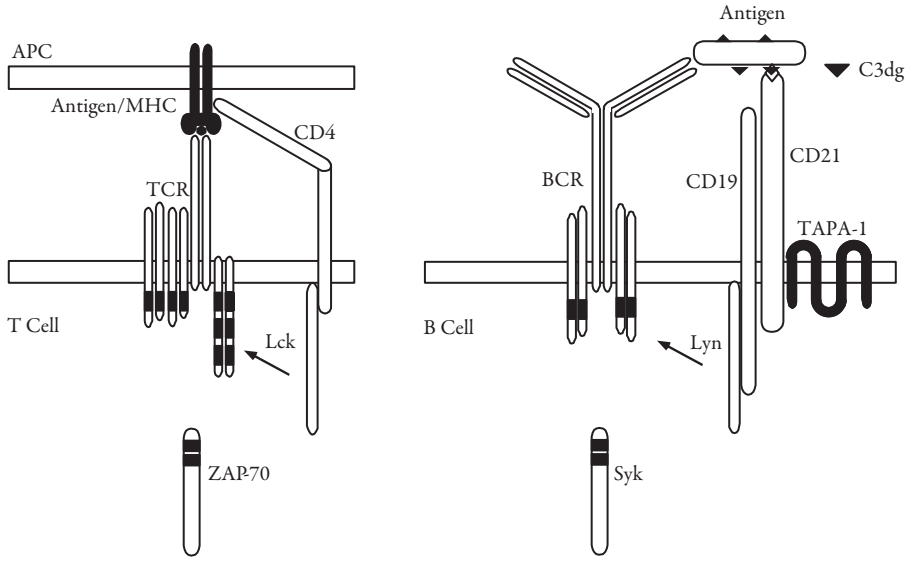


Figure 6.3 ***Models of co-receptor/antigen receptor engagement on T and B cells.*** CD19 also recruits downstream signaling components to its cytoplasmic domain, as discussed later in this review. APC, antigen-presenting cell; MHC, major histocompatibility complex.

motif shared by CD4 and CD8 (Chow and Veillette, 1995). This interaction is important but not essential for TCR signal transduction during development or at low antigen concentrations (Singer and Bosselut, 2004). Several functional consequences may result from the interaction of Lck with the CD4 and CD8 co-receptors. If CD4 or CD8 delivers Lck to the TCR complex through co-localization driven by the interaction of the co-receptors with the MHC/peptide complex, the phosphorylation of ITAMs by Lck would be facilitated and subsequent interactions involving the phosphorylation and activation of ZAP-70 or Syk could be potentiated (Weiss, 1993). In addition to this initiating function of Lck in TCR signal transduction, Lck appears to contribute to T-cell activation in a second way in which a CD4-bound Lck molecule binds to phosphorylated ZAP-70 or Syk via its SH2 domain, thereby stabilizing the interaction of the co-receptor with the TCR-MHC/peptide complex (Straus et al., 1996; Thome et al., 1995; Xu and Littman, 1993). However, there are some data suggesting that the CD4 complex that delivers Lck to the ITAMs may not be able to bind to the same MHC molecule as the TCR (Davis and van der Merwe, 2006). However, this does not exclude the notion that CD4 does help to concentrate Lck in the region of the TCR.

Mature CD4 or CD8 T cells are derived from thymocyte precursors that express both of these coreceptors. Evidence suggests that these two co-receptors play an active role in the decision process regarding lineage commitment (Singer et al., 2008). Moreover, T cells that express CD4 or CD8, in addition to recognizing peptide antigens on distinct classes of MHC molecules, generally have distinct effector functions, helper or cytolytic functions, respectively. To explain this linkage of co-receptor expression to differentiated phenotype, it was initially proposed that the engagement of the TCR with each of these co-receptors leads to distinct signaling events. The identity of such specialized events remains unknown, and subsequent work has provided evidence instead for two distinct types of models that base lineage choice on quantitative levels of co-receptor-dependent TCR signaling. The first model, based on the fact that the interaction of CD4 with Lck appears to be more avid than that of CD8 with Lck, postulates that a higher level of TCR signaling during positive selection favors CD4 lineage development, whereas a lower level of TCR signaling favors CD8 lineage development (Hogquist, 2001). The second model postulates that the lineage decision is informed by the nature of temporal changes in the level of TCR signaling, as $CD4^+$ $CD8^+$ thymocytes undergoing positive selection downregulate CD8 in the transition to becoming a single positive thymocyte (Singer et al., 2008). According to the latter model, if TCR signaling continues, the cell becomes a CD4 T cell, but if positive selection signaling ceases, the cell then re-expresses CD8 and turns CD4 expression off, and if TCR signaling resumes then the cell becomes a CD8 T cell.

CO-RECEPTORS ON B CELLS

Unlike most αβ TCR-expressing T cells, B cells do not recognize complexes of antigen-derived peptides with MHC molecules and hence do not need the CD4 or CD8 co-receptors. Nonetheless, B cells do express their own dedicated co-receptors and use these co-receptors to gain important information about the nature of an antigen. B-cell co-receptors substantially alter the magnitude and possibly alter the nature of the signaling by the BCR and hence can greatly affect activation of the B cell. B-cell co-receptors can promote activation of B cells, by indicating that the antigen is likely to be foreign, for example if it has been decorated with complement components, or they can inhibit activation to antigens that are linked to sialic acid-containing oligosaccharides or are coated with IgG antibodies. The former is likely an indication that the antigen may be a self-antigen, whereas the latter may indicate

that sufficient antigen-specific IgG has already been made and additional activation of naïve B cells is unnecessary.

Strong evidence for the ability of complement to tag an antigen and thereby promote antibody responses in vivo was obtained through the use of fusion proteins between lysozyme and one or more copies of the C3d fragment of complement, which is a ligand for complement receptor 2 (CR2) on B cells. Addition of a single C3d to lysozyme decreased the amount of protein needed to induce an antibody response by ten-fold, and addition of three tandem C3d fragments decreased the amount needed by more than 1,000-fold (Dempsey et al., 1996), demonstrating a strong enhancing function of CR2 for B-cell activation. This appears to require co-localization of the CR2 complex with the BCR, as shown by co-cross-linking studies. Interestingly, preligation of CD19, the signaling component of the CR2 complex, inhibits IgM-mediated B-cell proliferation in a manner that is remarkably similar to the effects on T-cell activation of preligating the T-cell coreceptors, CD4 and CD8.

CR2, also known as CD21, forms a molecular complex with CD19 and the tetraspanin CD81, also known as TAPA-1 (Fig. 6.3). CD19 and CD81 are thought not to have ligands of their own, although CD81 serves as an entry receptor for infection of cells by hepatitis C virus (Pileri et al., 1998). CD81 may serve to promote the association of the CR2 complex with membrane rafts (see below), whereas the cytoplasmic tail of CD19 provides the major signaling function to the complex. The enhancement of BCR signaling by CD19 appears to extend beyond CR2 engagement, since the B-cell phenotype of $CD19^{-/-}$ mice is more severe than the phenotype of $CD21^{-/-}$ mice. CD19 appears to promote BCR signaling in the absence of complement deposition on the antigen, particularly when the B cell contacts a membrane-bound antigen (Depoil et al., 2008), for example when antigen is held on the surface of a follicular dendritic cell in a germinal center reaction, or when it is held on the surface of a macrophage or dendritic cell in the secondary lymphoid organs. The positive role of CD19 in B-cell activation is additionally illustrated by the striking effects of increasing CD19 expression via a transgene in mice, which results in an abundance of highly activated B cells (Engel et al., 1995).

CD19 is thought to promote BCR signaling by several mechanisms, including binding of the signaling components phosphoinositide 3-kinase (PI 3-kinase) and Vav, and binding of the Src-family PTK Lyn. Phosphorylation of CD19 on its two YxxM motifs (Y482 and Y531) results in recruitment of PI 3-kinase via the SH2 domain of the p85 regulatory subunit (Tuveson et al., 1993). This occurs when CD19 is co-localized with a signaling BCR and its activated protein tyrosine kinases. Mutation of the PI 3-kinase binding sites in the YxxM motifs of CD19 results in a strong defect in the ability of B cells to undergo a successful germinal center reaction, leading to production of high-affinity class-switched antibodies (Wang et al., 2002; Wang and Carter, 2005). The role of CD19 in humans is likely to be similar to what has been reported in mice since defects in CD19 result in common variable immunodeficiency, the main hallmark of which is a defective germinal center response (Schaffer et al., 2007).

The important role of the YxxM motifs in the cytoplasmic tail of CD19 likely reflects the central role of PI 3-kinase in BCR signaling, as is discussed in more detail below. However, it should be noted that Lyn also binds to one of these sites (Y531), complicating the interpretation of mutations of this site. Recruitment and activation of Vav is also likely to be important for the ability of CD19 to promote recognition of membrane-bound antigens (Arana et al., 2008; Weber et al., 2008). The importance of Lyn association with CD19 is less well understood, but by analogy with the role of CD4 and CD8 in TCR signaling, described above, it is attractive to think that this contributes to the great sensitivity of the B cell to lysozyme-C3d fusion proteins observed in vivo.

CORECEPTORS WITH INHIBITORY FUNCTIONS

Inhibitory receptors are now recognized to play critical roles in restraining activation of immune cells by ITAM-containing receptors (Ravetch and Lanier, 2000). Many of these receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which become phosphorylated when the inhibitory receptor is brought adjacent to an activating receptor with ITAMs and then recruits one or more SH2-containing phosphatases that attenuate the signaling. In the case of B cells, two inhibitory receptors, FcγRIIB and CD22, appear to function to inhibit BCR signaling when the antigen is decorated with their ligands and hence can mediate their juxtaposition with the antigen-engaged BCR. Thus, they function analogously to the positively acting coreceptors described above, except that they function oppositely to inhibit antigen receptor signaling.

The best understood of these inhibitory receptors is FcγRIIB. Co-ligation of this receptor with the BCR inhibits B-cell proliferative responses and promotes B-cell apoptosis (Nimmerjahn and Ravetch, 2008). Physiologically, co-ligation is likely to occur during the binding of antigen–antibody complexes to antigen-specific B cells. The presence of IgG bound to the antigen reflects the presence of specific high-affinity antibody, so inhibition of further B-cell activation is a mechanism for downregulating antibody production late in an immune response. Indeed, mice made deficient in FcγRIIB have heightened B-cell responses to BCR stimulation, especially at later times after immunization (Takai et al., 1996). Co-ligation of the BCR with FcγRIIB leads to FcγRIIB tyrosine phosphorylation (Muta et al., 1994). The major inhibitory function of this receptor maps to a single tyrosine residue in its cytoplasmic domain that is in the context of the ITIM consensus sequence (I/L/VxYxxL). Phosphorylation of the tyrosine residue in the ITIM leads to the recruitment of the SHIP-1 inositol 5-phosphatase via its SH2 domains (Nimmerjahn and Ravetch, 2008). SHIP-1 modulates BCR signaling by removing the 5-phosphate from phosphatidylinositol 3, 4, 5 trisphosphate (PIP_3), a key molecule involved in BCR signaling, as described in detail below. In addition, SHIP forms a complex with another adaptor protein, $p62^{dok}$, which becomes strongly tyrosine phosphorylated upon BCR-FcγRIIB co-cross-linking and recruits RasGAP, a suppressor of Ras activity (Tamir et al., 2000; Yamanashi et al., 2000).

CD22 is a second cell-surface protein that attenuates signaling via the BCR in a proximity-dependent manner (Nitschke, 2005). CD22 is a transmembrane glycoprotein that belongs to the family of Siglecs (sialic acid binding Ig-like lectins) and is expressed exclusively on B cells. It has been proposed that CD22 acts as a co-receptor that inhibits activation of B cells recognizing cell-surface self-antigens, since such antigens would be expected to include sialic acid-containing oligosaccharides that could bind CD22 and recruit it to the BCR (Lanoue et al., 2002), where it can inhibit BCR signaling by ITIM recruitment of SHP-1 (SH2-containing protein tyrosine phosphatase 1) and SHIP-1. Accordingly, CD22-deficient B cells show augmented BCR-induced calcium and Erk MAP kinase responses (Nitschke, 2005). Moreover, sequestering CD22 away from the BCR by preincubation with anti-CD22-coated beads leads to robust mitogen-activated protein kinase (MAPK) activation upon BCR stimulation, whereas co-ligating BCR with CD22 suppresses MAPK activation.

Alternatively or additionally, CD22 associates with ligands on the surface of the B cell, and this association promotes its inhibitory function. In this way, CD22 may act as a rheostat that attenuates BCR signaling in a manner that is dependent on developmental maturity and/or activation state. In agreement with this view, recent studies have shown that the inhibitory effect of CD22 on BCR signaling is much stronger in mature resting B cells than it is on immature B cells of the bone marrow or their counterparts in the spleen, the transitional 1 (T1) and transitional 2 (T2) B cells (Gross et al., 2009). This developmental regulation of the inhibitory function of CD22 is due in part to a two- to three-fold upregulation of CD22 cell-surface expression on mature follicular B cells compared to T1 B cells, and may also be due to a change in the acetylation of B-cell-surface sialic acids that regulates their ability to bind to CD22 (Cariappa et al., 2009).

Upon BCR engagement in splenic B cells, most of which are mature, the cytoplasmic tail of CD22 is phosphorylated by the Src family kinase Lyn on six tyrosine residues. Three of these are in the context of ITIMs, in agreement with a negative regulatory role for CD22. Lyn-deficient mice lack BCR-induced tyrosine phosphorylation of CD22 and have reduced phosphorylation of FcγRIIB (Xu et al., 2005), arguing that Lyn is the primary tyrosine kinase involved in phosphorylation of the ITIMs of these inhibitory co-receptors. Interestingly, both Lyn-deficient and CD22-deficient mice spontaneously develop substantial titers of IgG anti-nuclear antibodies and anti-native DNA antibodies, autoantibodies characteristic of the autoimmune disease systemic lupus erythematosus. These results suggest that the Lyn/CD22/SHP-1 inhibitory pathway plays an important role in maintaining tolerance to nuclear autoantigens. Unknown is whether this reflects the proposed co-receptor role of CD22 or simply its ability to act as a rheostat that attenuates BCR signaling in mature follicular B cells, setting a higher threshold for B-cell activation.

BCR signaling can also be inhibited by the ITIM-containing cell-surface molecules CD72 and the paired immunoglobulin-like receptor B (PIR-B), although whether or not they are properly thought of as inhibitory coreceptors for the BCR is not known. B cells from CD72-deficient mice are hyperresponsive to suboptimal doses of antigenic and mitogenic stimulation and exhibit enhanced BCR signaling, which is consistent with an inhibitory coreceptor function (Li et al., 2006).

T cells also express a number of ITIM-containing inhibitory receptors, most of which are primarily expressed after antigen stimulation of naïve T cells. These include the inhibitory costimulatory receptors CTLA-4 and PD-1 and a variety of inhibitory receptors also expressed on NK cells. CTLA-4 (CD152) is closely related to the costimulatory receptor, CD28. Both bind to the counter-receptors, CD80 (B7-1) and CD86 (B7-2), which are upregulated on antigen-presenting cells. Antigen stimulation induces B7-2 on B cells, and innate immune stimuli induce expression of B7-1 and B7-2 on dendritic cells and macrophages. Whereas CD28 provides a second requisite signal, which together with that of the TCR can activate T cells, CTLA-4 has a negative regulatory function that serves to maintain a higher threshold for T-cell activation and can prevent T cells from making cytokines or proliferating to antigen. Its importance is highlighted by the lethal T-cell lymphoproliferative syndrome in mice when the *ctla-4* gene is inactivated (Fife and Bluestone, 2008).

CTLA-4 expression is upregulated in activated T cells and is relatively high in T regulatory cells. Exactly how CTLA-4 provides a negative regulatory function is not clear. The following mechanisms have been implicated: (1) competitive binding to CD80 and CD86 (CTLA-4 has a higher affinity for B7s than does CD28), thereby inhibiting the positive function of CD28; (2) downregulation of CD80 and CD86 on the surface of antigen-presenting cells; (3) binding of its cytoplasmic tail to PP2A, a protein serine-threonine phosphatase; and (4) binding of its cytoplasmic tail to SHP-2, a protein tyrosine phosphatase. These mechanisms all could mediate a direct inhibitory effect within effector T cells, but there is some evidence that CTLA-4 can provide inhibitory function in a cell non-autonomous fashion (Fife and Bluestone, 2008), which would fit with a function on regulatory T cells contributing to their inhibitory function. One striking feature of CTLA-4 that might provide a clue regarding its negative regulatory mechanism is that it rapidly recycles from the cell surface via endocytic pathways, maintaining a relatively low number of molecules on the cell surface.

PD-1 (CD279) is a cell-surface molecule expressed on some thymocyte subsets and on activated T cells. It has two ligands, PDL-1 and PDL-2; the expression of the former is fairly broad, while the expression of the latter seems limited to dendritic cells and monocytes. In peripheral T cells, PD-1 negatively regulates T-cell activation, and in chronically activated T cells, it can prevent further T-cell activation (Barber et al., 2006). It is inducibly tyrosine phosphorylated on two tyrosines, one of which is a classical ITIM motif (discussed above). It is known to interact with two cytoplasmic tyrosine phosphatases, SHP-1 and SHP-2 (Chemnitz et al., 2004; Okazaki et al., 2001). These phosphatases can then negatively regulate tyrosine phosphorylation-dependent events downstream of the TCR and thereby dampen or inhibit T-cell activation.

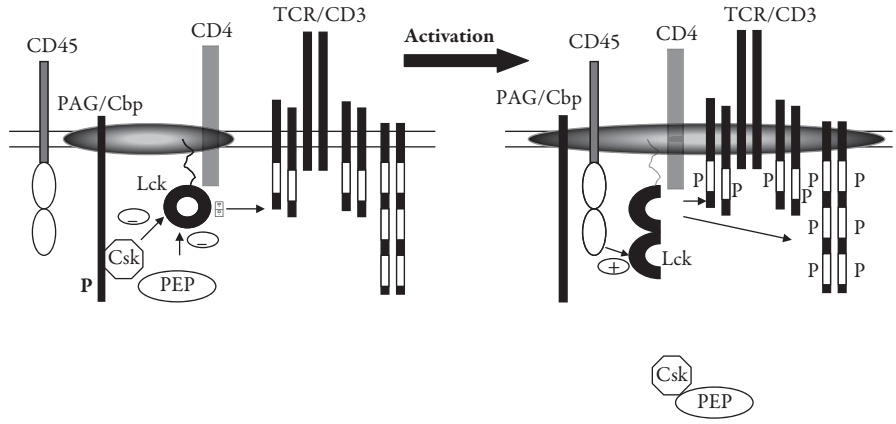


Figure 6.4 ***Dynamic regulation of Src family kinases by Csk and tyrosine phosphatases, CD45 and PEP, in plasma membrane microdomains (membrane rafts).*** According to one model that has received recent support, an early event in TCR signaling is dephosphorylation of PAG to release Csk and PEP from the plasma membrane, which promotes conversion of Src family kinases to a more highly activated form. This would positively amplify TCR signaling.

REGULATION BY CD45 AND CSK

The function of Src PTKs is tightly regulated by tyrosine phosphorylation (Cooper and Howell, 1993), which in turn is the result of a dynamic equilibrium involving the opposing actions of kinases and phosphatases. Phosphorylation of a tyrosine near the catalytic site of the kinase domain is associated with the activated state of these kinases. In addition, the C-terminal regulatory region of the Src kinases contains a tyrosine residue whose phosphorylation negatively regulates their activity. In T and B cells, the phosphorylation of this negative regulatory site in Src PTKs appears to be regulated by the opposing actions of the transmembrane PTP CD45 (as well as the transmembrane PTP CD148, in B cells; Hermiston et al., 2009) and the cytoplasmic PTK Csk (Fig. 6.4) (Chow and Veillette, 1995). The regulation of Lck in T cells by CD45 and Csk has been most thoroughly studied.

Various isoforms of CD45, derived by alternative splicing of exons 4 to 6, which encode portions of the extracellular domain, are expressed in a cell type- and activation-specific manner on all nucleated cells of the hematopoietic lineage (Hermiston et al., 2009; Lynch, 2004). Although the regulated expression of specific isoforms of CD45 suggests that CD45 may bind or be influenced by extracellular ligands, definitive identification of such ligands has not yet been achieved. As described above for CD22, it is also possible that the extracellular domain of CD45 interacts laterally with other molecules present in the same plasma membrane, thereby delivering the phosphatase domains to their targets. Some evidence of this has been obtained for interaction of the low-molecular-weight isoforms of CD45 with CD4 and the TCR (Dornan et al., 2002). Like most transmembrane PTPases, CD45 contains two tandem PTP homology domains in its cytoplasmic domain. Only the membrane proximal PTP domain clearly has catalytic phosphatase function, although the membrane distal domain may play an important regulatory function since it appears to be necessary for PTP activity of CD45 (Desai et al., 1994).

CD45 expression is required for normal T-cell development in mice (Kishihara et al., 1993). Interestingly, B-cell development is not as dependent on CD45, although the B cells that do develop in CD45-negative mice show functional defects. Another receptor-like PTP, CD148, which is not expressed on most thymocytes or naïve T cells, is expressed constitutively throughout B-cell and myeloid-cell development and appears to provide at least partially redundant phosphatase function with CD45 in the B-cell lineage (Zhu et al., 2008). In peripheral mature T and B cells as well as cell lines and clones, CD45, together with CD148 in the case of B cells, is required for TCR- and BCR-induced signal transduction (Hermiston et al., 2009). The PTP catalytic function of CD45 is required for this molecule to support TCR signal transduction. In its absence, the earliest phosphorylation of ITAMs or recruitment of ZAP-70 does not occur. In CD45-deficient T-cell lines, although there is not a substantial increase in basal tyrosine phosphorylation of most proteins, the negative regulatory tyrosine phosphorylation sites of Lck and Fyn are hyperphosphorylated (Hermiston et al., 2009; Ostergaard, Shackelford et al., 1989). Likewise, the Lyn negative regulatory site is hyperphosphorylated in B cells from CD45 and CD148 doubly deficient mice (Zhu et al., 2008). Phosphorylation of this site results in an inactive state of these PTKs because of a conformational constraint imposed by an intramolecular interaction between the C-terminal phospho-tyrosine and the SH2 domain of the kinase (Sicheri et al., 1997). Accordingly, Src family kinases are largely inactive in CD45-deficient lymphocytes. The observations made in CD45-deficient cells are consistent with the notion that the phosphotyrosines in the C-terminal regions of Src family kinases are physiological substrates of CD45 and CD148. Moreover, the greatly decreased activity of Lck and Fyn can account for the defect in TCR signal transduction function. Thus, in normal resting cells where these kinases are mostly dephosphorylated at the C-terminal-negative regulatory sites, CD45 and CD148 maintain the Src kinases in an antigen receptor-responsive but not fully active state. Activation of Src family kinases involves the phosphorylation of a critical tyrosine within the regulatory loop of the kinase domain in addition to the lack of phosphorylation of the negative regulatory site.

Observations of isoform-specific effects of CD45 in reconstituted T-cell hybridoma systems are suggestive of specific functional properties for the different isoforms (Novak et al., 1994). Studies with an epidermal growth factor receptor-CD45 chimera suggest that ligand-induced dimerization may negatively regulate the phosphatase function of CD45 (Desai et al., 1993). Inactivation by dimerization of CD45 appears to be mediated by a putative wedge-like structure in the juxtamembrane region that blocks the catalytic site of the partner molecule during dimerzation (Majeti et al., 1998). Supporting the significance of this regulatory mechanism, disruption of the wedge function in mice leads to lymphoproliferation and autoimmunity (Majeti et al., 2000). Interestingly, the different splice variants of CD45 have differences in their affinities for homodimerization, likely affecting the overall activity of CD45 in the cell (Xu and Weiss, 2002). Thus, the dimerization of CD45 is a regulated event that affects its activity and thereby restrains lymphocyte activation.

The PTK responsible for the phosphorylation of the negative regulatory sites of Src PTKs is Csk, a widely expressed PTK that contains SH2 and SH3 domains (Chow and Veillette, 1995). In the basal state, Csk and CD45 are presumably in equilibrium, maintaining the C-terminal tyrosines of Src family protein kinases primarily in the unphosphosphorylated form. Overexpression of Csk can inhibit TCR-induced protein tyrosine phosphorylation and interleukin-2 (IL-2) production. Regulation of Csk occurs by its differential distribution between the cytosol and lipid raft fraction of the plasma membrane during lymphocyte activation (Fig. 6.4). The lipid raft resident transmembrane adaptor protein PAG1 was identified as a binding partner for Csk in resting T cells (Brdicka et al., 2000). PAG1 is constitutively tyrosine phosphorylated in resting T cells and binds to Csk via the SH2 domain of the latter. Following TCR cross-linking, PAG1 is dephosphorylated by an unknown tyrosine phosphatase, thereby releasing Csk, which relocalizes to the cytosol, allowing the balance to be shifted in the favor of Src family kinase activation (Davidson et al., 2003). This could be part of a positive feedback loop, whereby release of the negative regulator Csk from the membrane results in diminished phosphorylation of Src kinases on their negative regulatory sites. Later, PAG1 gets rephosphorylated and recruits Csk back to the lipid microdomains, where it can exert its negative regulatory effect on Lck and Fyn once again. Surprisingly, mice deficient in PAG1 have little phenotypic abnormality (Dobenecker et al., 2005). However, this may simply reflect the presence of proteins with functions redundant with that of PAG1. For instance, the LIME protein has been shown to interact with Csk and Lck (Brdickova et al., 2003; Hur et al., 2003).

In addition to its role in phosphorylating the negative regulatory C-terminal tyrosine of Src family kinases, Csk also negatively regulates TCR signaling by associating with an intracellular protein tyrosine phosphatase, proline-enriched phosphatase (PEP) (Hermiston et al., 2009), which is expressed in hematopoeitic cells. PEP has been found to associate with Csk via the SH3 domain of Csk (Veillette et al., 2009). Substrate-trapping experiments using mutant PEP identified Zap-70 and FynT as two proximal targets of PEP-mediated inhibition and the site of dephosphorylation on FynT was mapped to the positive regulatory tyrosine 417. Thus, a complex of Csk–PEP acts synergistically to interfere with TCR signaling by phosphorylating the C-terminal negative regulatory tyrosine and dephosphorylating the activating tyrosine of Src family kinases (Cloutier and Veillette, 1999). Mice made deficient in PEP have a rather mild phenotype, with increased T-cell proliferative responses during secondary responses (Hasegawa et al., 2004). In addition to PEP, two other members of the PEP family, PTP-PEST and PTP-HSCF, have been reported to cooperate with Csk in the regulation of antigen-receptor signaling in T cells (Veillette et al., 2009). Whether PEP, PTP-PEST, and PTP-HSCF all act redundantly or uniquely is currently unknown. Nonetheless, the importance of PEP in the immune system is indicated by the association of a moderately common allele of PTPN22 (the gene for the human homolog of PEP) with increased susceptibility for several autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus (Vang et al., 2007). The amino acid change seen in this allele disrupts PTPN22's association with Csk, indicating that this association is important for maintaining tolerance to self. This is consistent with the reduced inhibition of TCR signal transduction by Csk and the allele of PTPN22 that does not associate with Csk (Zikherman et al., 2009).

Genetic analysis in model organisms also supports the view that Csk plays an important role in regulating lymphocyte function. In Csk-deficient chicken B cells, Lyn was found to be highly phosphorylated at the autophosphorylation site and constitutively active. In addition, Syk was also constitutively activated, to an extent similar to that observed upon BCR stimulation. However, BCR cross-linking was still required for other cellular proteins to be tyrosine phosphorylated and for inositol 1, 4, 5-trisphosphate (IP_3) generation and calcium mobilization (Hata et al., 1994). Homozygous mutant mice with a disruption in the Csk gene were found to have neural tube defects, were embryonic lethal, and died during gestation at days 9 to 10. The Csk-deficient embryonic cells exhibited an increase in the activity of Src, Fyn, and Lyn kinases, which suggests that Csk regulates the activity of these kinases; this may be essential during embryogenesis (Imamoto and Soriano, 1993). The role of Csk in lymphocyte development was studied by conditionally inactivating Csk in immature thymocytes. Lack of Csk was found to override the requirement for pre-TCR, αβ TCR, and MHC class II for the development of $CD4^+CD8^+$ double-positive and $CD4^+$ single-positive thymocytes as well as peripheral CD4 αβ T-lineage cells (Schmedt et al., 1998; Schmedt and Tarakhovsky, 2001). Thus, in the absence of negative regulation of the Src family kinases, these kinases can drive the development of αβ T cells independently of the pre-TCR and the αβ TCR. In support of this conclusion, triple knockout mice lacking Csk, Lck, and Fyn show a block in the development of αβ T cells, indicating that Lck and Fyn are responsible for driving T-cell development in the absence of Csk. Furthermore, Csk was shown to play a differential role in positive and negative selection during development (Schmedt and Tarakhovsky, 2001).

SIGNALING PATHWAYS ACTIVATED BY ANTIGEN RECEPTOR-INDUCED TYROSINE PHOSPHORYLATION

Stimulation of the TCR and BCR induces the tyrosine phosphorylation of many intracellular proteins and sets into motion a number of signaling pathways. In the following sections, we will focus on some of the better-understood TCR- and BCR-regulated signaling pathways and discuss the involvement of some of these proteins in lymphocyte activation.

ROLE OF ADAPTOR PROTEINS

Signal transduction via the TCR and BCR flows from the membrane proximal tyrosine kinases to downstream signaling events such as activation of signaling enzymes, opening of endoplasmic reticulum (ER) and cell surface calcium channels, activation of small GTPases of the Ras superfamily, conversion of cell-surface integrins to their high-affinity state, rearrangement of the actin cytoskeleton, and changes in the activity of a variety of transcriptional regulators. The transmission of these signals requires the formation of multimolecular complexes consisting of signaling components with adaptor molecules that contribute to the specificity and localization of signaling events. Adaptor proteins are those signaling components that lack enzymatic or transcriptional activity, but instead act to bring together other signaling components or bring them to the plasma membrane to promote their functions. Adaptors often comprise modular domains such as the SH2, SH3, PTB, PDZ, or PH domains or simply multiple tyrosines or proline-rich motifs that can act as docking sites for some of the above domains (Pawson, 1995; Pawson and Nash, 2000).

Some adaptors are membrane proteins whereas others are located in the cytosol in unstimulated cells and are recruited to the plasma membrane by initial receptor signaling events. In the former category are the transmembrane adaptors LAT, which is required for TCR signaling, and CD19, which is part of the complement receptor 2 co-receptor complex in B cells and also participates in BCR signaling in the absence of complement tagging of the antigen. Both proteins contain multiple tyrosine residues that are phosphorylated upon receptor ligation and form docking sites for other downstream adaptors and signaling enzymes and therefore serve to nucleate the formation of multimeric signaling complexes, or "signalosomes."

TCR-associated and activated ZAP-70 phosphorylates LAT, which then recruits the signaling enzyme phospholipase Cγ1, the Ras-activator mSOS via its association with the SH2 domain-containing adaptor Grb2, and the cytosolic adaptor SLP-76 via its association with a related SH2 domain-containing adaptor Gads (Samelson, 2002). SLP-76 in turn nucleates a complex with other signaling molecules, including the cytoplasmic protein tyrosine kinase Itk and the Rho family GEF Vav (Yablonski and Weiss, 2001). Several molecules related to LAT have been identified, including LAB (also called NTAL), LAX, and LIME (Horejsi et al., 2004). Although these molecules are also localized in the plasma membrane and have multiple sites of phosphorylation, none seems to play as central a role in immunoreceptor signaling as LAT.

The connections between antigen receptor-activated tyrosine kinases and signaling reactions in B cells is only analogous to T cells in some ways but different in others. No single transmembrane adaptor analogous to LAT that is essential for BCR signaling has been found, and it is likely that there is no such adaptor. B cells do express a relative of SLP-76 called B-cell linker protein (BLNK) (also called SLP-65 or BASH) (Wollscheid et al., 1999). SLP-76 and BLNK have similar domain structures and recruit several of the same signaling molecules in T and B cells. Both SLP-76 and BLNK have a single C-terminal SH2 domain, which recruits them from the cytosol to the plasma membrane when signaling is initiated. As mentioned above, SLP-76 is recruited to LAT. In contrast, BLNK is recruited directly to the BCR, binding to a phosphorylated non-ITAM tyrosine (Y204) in the Igα cytoplasmic domain (Engels et al., 2001; Kabak et al., 2002; Patterson et al., 2006). There is likely an alternative mechanism of recruitment of BLNK to the plasma membrane, possibly involving direct interaction with the lipid bilayer (Kohler et al., 2005), since mice with specific mutation in Y204 of Igα have a less severe B-cell phenotype than mice lacking BLNK altogether (Patterson et al., 2006).

SLP-76 and BLNK have multiple tyrosines that are phosphorylated upon stimulation and thereby contribute to signaling-dependent formation of multicomponent signaling complexes at the plasma membrane adjacent to signaling antigen receptors. Other protein–protein interaction domains and motifs that are present in these proteins also contribute to the formation of these signaling complexes.

In cells lacking SLP76 or BLNK, proximal receptor engagement is uncoupled from distal events such as calcium flux and IL-2 production (Clements et al., 1998; Pappu et al., 1999; Yablonski et al., 1998). Deficiency of SLP-76 or BLNK in mice leads to strong defects in T- and B-cell development, respectively (Clements et al., 1998; Hayashi et al., 2000; Pappu et al., 1999). BLNK also plays an essential role in BCR signaling in humans, and genetic deficiency in this component leads to a severe agammaglobulinemia (Minegishi et al., 1999), similar in phenotype to a Btk deficiency but much less common because BLNK is encoded on an autosomal chromosome rather than the X chromosome (Chapter 25).

As mentioned above, CD19 also contributes to BCR signaling some of the functions provided by LAT and SLP-76. Mice genetically defective in CD19 have impaired B-cell development (Engel et al., 1995), indicating the importance of the contribution of CD19 to BCR signaling.

THE PHOSPHATIDYLINOSITOL-BASED SIGNALING PATHWAYS

Two of the central signaling reactions triggered by antigen receptors utilize enzymes acting on the plasma membrane phospholipid phosphatidylinositiol 4,5-bisphosphate (PIP_2) to generate second messengers, which in turn activate a variety of signaling reactions. PIP_2 is acted upon either by PI 3-kinase, which adds a third phosphate to the inositol head group,

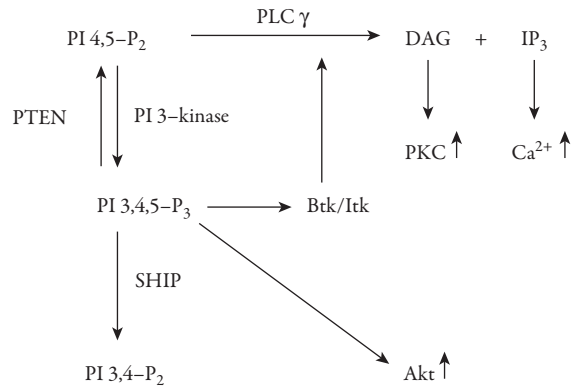


Figure 6.5 ***Critical signaling events involving phosphatidylinositol-containing lipids.*** Both the PI 3-kinase signaling pathway and the phosphoinositide hydrolysis signaling pathway emanate from PIP2, as shown. PI, phosphatidylinositol; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; IP3, inositol trisphosphate; SHIP, SH2 domain-containing inositol phosphatase.

generating phosphatidylinositol 3,4,5-trisphosphate (PIP_3), or by phospholipase Cγ1 (PLC-γ1) in T cells or PLCγ2 in B cells, both of which hydrolyze PIP_2 to yield the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglyercol (DAG) (Fig. 6.5). While it was initially thought that these were two independent signaling reactions emanating from the same precursor, subsequent studies have shown a more hierarchical signaling architecture in which PIP_3, in addition to promoting unique signaling events, contributes importantly to the activation of PLC-γ. The latter role appears to be more pronounced in B cells than in T cells (Fruman and Bismuth, 2009).

The product of PI 3-kinase, PIP_3 serves two major signaling functions. It serves as a binding site for several signaling molecules that contain pleckstrin homology (PH) domains, including the Tec family tyrosine protein kinases Itk (T cells) and Btk (B cells), and PLC-γ, thereby directing their localization within the cell. In addition, PIP_3 promotes activation of the protein kinases PDK1 and Akt. Akt has a PH domain that binds PIP_3, which allows Akt to be recruited to the membrane in response to PIP_3 elevation. Once at the membrane, Akt is activated by a second kinase, PDK-1 (3-phosphoinositide-dependent protein kinase 1). Activated Akt in turn promotes survival, inhibits the quiescence-promoting transcription factors of the FOXO subfamily (Fruman and Bismuth, 2009), and promotes glucose uptake and metabolism (Jones and Thompson, 2007), all of which contribute to antigen-induced activation of mature lymphocytes (Fig. 6.5). The biological importance of this pathway is illustrated by the promotion of autoimmunity and lymphoproliferation in the mouse by loss-of-function mutations of PTEN, which enhance PI 3-kinase signaling by decreasing hydrolysis of PIP_3 by PTEN, and conversely by the immunodeficiency that results from targeted disruption of PI 3-kinase subunits in B cells or T cells (Fruman and Bismuth, 2009).

The PI 3-kinase isoforms activated by antigen receptors are class IA PI 3-kinases containing a 110 kDa catalytic subunit (mostly the p110δ isoform, although T cells also express the p110α and p110β isoforms), with an SH2 domain-containing regulatory subunit of either 85 kDa (p85α or p85β) or of 50 or 55 kDa (p50α, p55α, or p55γ). The larger isoforms of the PI 3-kinase regulatory subunit contain additional protein–protein interaction domains (a SH3 domain and a RhoGAP-like domain) lacking in the small forms, but the contributions of these domains to lymphocyte signaling are not understood. Mice with genetic ablation of p85α or p110δ, the main subunits of PI 3-kinase in B cells, exhibit impaired maturation of developing B cells and poor B-cell activation via the BCR, defects that are very similar to those seen in mice deficient in Btk (Fruman and Bismuth, 2009). Moreover, in both the Btk mutant B cells and the B cells with defective PI 3-kinase, there is defective BCR-induced calcium elevation, due to substantially impaired PIP_2 hydrolysis by PLC-γ. It is currently thought that this reflects the BCR-induced formation of a multimolecular complex including BLNK, Btk, and PLC-γ2, which fails to localize properly to the plasma membrane in the absence of binding of the PH domains of Btk and/or PLC-γ2 to PIP_3 in the membrane. In comparison, PIP_2 hydrolysis downstream of the TCR is less dependent on PIP_3 in the membrane. The reasons for this difference between B cells and T cells are not well established, but possible explanations include the fact that there is a transmembrane adaptor (LAT) that recruits PLC-γ1 to the plasma membrane, and that Tec family tyrosine kinases in T cells include, in addition to an isoform that must be recruited to the plasma membrane (Itk), an isoform that is constitutively tethered to the membrane (Rlk). In any case, PIP_3 plays an important role in promoting T-cell activation through its critical role in activating PDK1 and Akt.

Whereas both the BCR and the TCR directly activate the PI 3-kinase signaling pathway, co-receptors or co-stimulatory receptors amplify or inhibit this pathway to influence lymphocyte activation. In B cells, the positively acting CD19 and the negatively acting FcγRIIB act primarily to increase or decrease, respectively, the amount of PIP_3 formed in the membrane in response to BCR engagement. The cytoplasmic domain of CD19, which is the signaling component of the CR2 coreceptor complex and also contributes to BCR signaling in the absence of complement involvement, contains two copies of the YxxM motif, which matches the consensus for binding by the two SH2 domains of the PI 3-kinase regulatory subunits, and these motifs are required for recruitment of PI 3-kinase to CD19 and its participation in BCR signaling. PI 3-kinase activation in B cells is additionally supported by a cytosolic adaptor protein called B-cell adaptor for PI 3-kinase (BCAP) (Kurosaki and Hikida, 2009). Signaling downstream of PI 3-kinase is greatly attenuated by the negative coreceptor FcγRIIB, the ITIM of which recruits selectively SHIP-1 (SH2-containing polyphosphate inositol 5-phosphatase), which removes the 5-phosphate from PIP_3, generating phosphatidylinositol 3,4-bisphosphate, an isomer of the form of PIP_2 that is the starting point for both PI 3-kinase and PLC-γ signaling pathways. The form of PIP_2 generated by SHIP-1 is a ligand for the PH domains of some signaling components, but its function is not well understood. The major effect of SHIP-1 is thought to be reduction of PIP_3 in the plasma membrane and attenuation of PIP_3-dependent signaling events. Correspondingly, a major effect of co-ligation of FcγRIIB with

the BCR is to greatly attenuate PIP_2 hydrolysis and calcium elevation. Thus, both the positively acting CR2 coreceptor and the negatively acting FcγRIIB coreceptor primarily work through modulation of the PI 3-kinase signaling pathway.

In T cells, it is co-stimulatory receptors rather than co-receptors that modulate PI 3-kinase signaling. Co-stimulatory receptors recognize ligands on antigen-presenting cells and either promote T-cell activation or inhibit it, depending on the receptor. On naïve, resting T cells, CD28 plays a critical co-stimulatory function in T-cell activation by binding CD80 and CD86 (B7-1 and B7-2), the expression of which is greatly upregulated on antigen-presenting cells by Toll-like receptors and receptors for inflammatory cytokines. CD28 costimulation can lead to increased cytokine production, enhanced proliferation, and diminished apoptosis in TCR-mediated responses (Salomon and Bluestone, 2001). The cytoplasmic domains of CD28 contains a YxxM motif for recruiting PI 3-kinase and boosting this signaling reaction. Depending on the model system used, the PI 3-kinase binding motif in the cytoplasmic tail of CD28 is required to mediate the co-stimulatory effect being measured or is unimportant, with several proline-rich putative SH3-binding motifs implicated in promoting alternative signaling pathways (Parry et al., 2007). One downstream effector of the PI 3-kinase pathway, the Akt serine/threonine kinase, can partially substitute for CD28 signals in T-cell lines and in CD28-deficient T cells (Kane et al., 2001), supporting the importance of this pathway in primary T cells. ICOS, a second co-stimulatory receptor that plays a critical role in promoting T-cell help for antibody responses, also recruits PI 3-kinase as part of its stimulatory function (Dong et al., 2003). In addition, two inhibitory members of the co-stimulatory receptor family, CTLA-4 and PD-1, have been reported to attenuate Akt activation, but the biochemical mechanisms of these effects are not well established, nor is the importance of this regulation for their inhibitory function. Thus, it appears that a significant mechanism by which co-stimulatory receptors affect T-cell activation is via modulation of the PI 3-kinase signaling pathway, but further studies are needed to establish this point.

A second key signaling reaction starting from PIP_2 is the hydrolysis of this molecule by PLCγ1 or PLCγ2. PLCγ2 is preferentially expressed in B cells, whereas PLCγ1 is preferentially expressed in T cells. Hydrolysis of PIP_2 by these enzymes generates IP_3, which is responsible for elevation of calcium levels in the cytoplasm, and diacylglycerol (DAG), which promotes activation of protein kinase C (PKC) isozymes and activation of the Ras activator, RasGRP. Following TCR or BCR stimulation, PLCγ isozymes translocate to the membrane, where they become tyrosine phosphorylated on critical tyrosine residues, increasing their catalytic activity. This translocation involves the recruitment of PLCγ isozymes via their SH2 domains to phosphorylated tyrosine residues on LAT in T cells and BLNK in B cells. Moreover, the SH3 domain of PLCγ1 interacts with a proline-rich motif in SLP-76, which may explain the requirement of this adaptor for PLC activation (Yablonski et al., 2001).

Stimulation of the TCR and BCR results in a rapid and sustained increase in $[Ca^{2+}]_i$. The immediate increase in $[Ca^{2+}]_i$ is the result of the release of intracellular stores of calcium through the action of IP_3 on IP_3 receptors in the ER (Sugawara et al., 1997). Release of the intracellular stores of calcium, however, results in only a transient rise in $[Ca^{2+}]_i$. The sustained increase requires an inward transmembrane flux of extracellular calcium ("store-operated calcium entry"), which occurs through plasma membrane Ca^{2+} channels that contain the ORAI1 calcium channel. ORAI1 was identified in an siRNA screen carried out with a Drosophila cell line, and, in addition, a mutation in this protein was discovered to be the cause of a defective calcium response in T cells from a patient with severe combined immunodeficiency (Feske et al., 2006); see also Chapter 20. Similarly, ORAI1 deficiency in mice results in immunodeficiency (Oh-hora and Rao, 2008). The human genome has two close homologs of ORAI1, called ORAI2 and ORAI3, but ORAI1 is the major isoform expressed in T cells. The "calcium-release activated calcium" (CRAC) channels containing ORAI1 are regulated by a transmembrane ER protein STIM1, which contains an EF hand, a sensor for calcium levels, in the part of the protein localized to the inside of the ER (Liou et al., 2005). A second related protein, STIM2, is also present in lymphocytes but has a different calcium sensitivity and seems to play a greater role in maintaining calcium elevation at later times and also in maintaining basal cytoplasmic calcium levels in the absence of TCR stimulation (Brandman et al., 2007). When calcium in the ER is depleted in response to IP_3 levels in the cytoplasm, STIM1 oligomerizes and now appears by fluorescence microscopy to co-localize with ORAI1 in areas of the cell where the ER and the plasma membrane come very close to one another (Oh-hora and Rao, 2008). Evidence that ORAI1 and STIM1 interact directly has recently been reported (Park et al., 2009). This STIM1/ORAI1 interaction maintains elevated intracellular free calcium levels in the cytoplasm as long as PIP_2 hydrolysis continues because IP_3 prevents refilling of the ER with calcium by maintaining open calcium channels in that organelle. When PIP_2 hydrolysis ceases, the ER refills and STIM1 dissociates from ORAI1, which no longer allows calcium entry across the plasma membrane. These changes, combined with mechanisms that pump calcium out of the cytoplasm, return the level of cytoplasmic calcium to that of the unstimulated state. The sequence of events in B cells is thought to be very similar.

The increase in $[Ca^{2+}]_i$ triggered by the TCR or BCR has effects on several downstream events, including the activation of calcium/calmodulin-dependent kinase II and calcineurin, a calcium/calmodulin-regulated protein serine/threonine phosphatase (also called PP2B). These events inhibit migration of T cells, promoting stable interactions with antigen-bearing antigen-presenting cells, promote degranulation of cytotoxic T cells, and promote the activity of a series of transcription factors. The activation of calcium/calmodulin-dependent kinase II has been implicated in T-cell anergy (Nghiem et al., 1994) and in activation of the CREB transcription factor, whereas the function of calcineurin has received considerably greater attention. Calcineurin function has been implicated in T- and B-cell responses that depend on TCR and BCR functions in a wide variety of experimental systems. This connection was made possible through the use of the immunosuppressives

cyclosporin A and FK506, which, when bound to their cellular receptors (cyclophilin and FKBP, respectively), function as specific inhibitors of calcineurin (Crabtree, 1999). Among the best examples illustrating the importance of calcineurin in lymphocyte responses are the studies of its role in the activation of a family of transcriptional factors, nuclear factor of activated T cells (NFAT), which plays an important role in the regulation of most cytokine genes, including IL-2. Calcineurin catalytic function has been shown to be required for the transformation of inactive cytoplasmic NFAT into active nuclear NFAT, where it interacts with AP-1 transcription factors (made up of Fos and Jun family members) at composite elements in the upstream IL-2 regulatory region (Crabtree, 1999). This requirement involves dephosphorylation of the inactive cytoplasmic NFAT, exposing its nuclear localization sequence (Oh-hora and Rao, 2008). Thus, by virtue of its regulatory influence on multiple cytokine genes, an increase in NFAT activity resulting from a calcium elevation can have profound effects on the subsequent immune response.

The other second messenger released by PIP_2 hydrolysis is DAG, which is an activator of RasGRP, which activates Ras, and also is an activator of protein kinase C (PKC) isozymes. Some PKC isoforms expressed in lymphocytes are calcium- and DAG-dependent enzymes (PKCα, β1, and β2), whereas others are DAG-dependent but calcium-independent (PKCε, δ, η, and θ) (Baier and Wagner, 2009). These functions of DAG can be mimicked by addition to cells of the much more stable pharmacological activators of the phorbol ester family. While different PKC isoforms likely have many functions, clearly two important functions are in the activation of the transcription factor NF-κB and in the activation of Ras, as described below.

SIGNALING PATHWAY LEADING TO ACTIVATION OF NF-κB

Lymphocyte survival and activation are highly dependent on NF-κB function (Gerondakis et al., 2006). Recent studies have greatly increased our understanding of the signaling pathway connecting antigen receptors to NF-κB activation. NF-κB is typically composed of a heterodimer of p50 and either p65 (also called RelA) or c-Rel, although homodimeric forms are known, as is an alternative complex made up of p52 and RelB, which is activated by some TNF receptor superfamily members. A small family of inhibitors, collectively called I-κB, maintain NF-κB in an inactive state until the IκB kinase (IKK) complex becomes activated. Active IKK phosphorylates I-κB, which leads to its ubiquitinylation and degradation by the proteasome. This releases NF-κB, which then translocates to the nucleus and activates transcription of NF-κB-responsive genes. NF-κB plays a key role in the induction of many genes involved in inflammation and immunity, including many cytokines, chemokines, and intracellular survival factors of the Bcl-2 family (Sen, 2006).

Essential to BCR- and TCR-induced NF-κB activation are three proteins that form a complex following antigen receptor engagement, CARMA1, Bcl10, and MALT1. Mice defective in any of these components have severe defects in antigen receptor-induced NF-κB activation but still activate NF-κB downstream of TNFR superfamily members or downstream of Toll-like receptors (Rawlings et al., 2006). CARMA1 is a large scaffold protein with multiple protein–protein interaction domains that regulate membrane localization, oligomerization, and Bcl10 binding. Recent genetic and biochemical studies support the hypothesis that select protein kinase C isoforms, PKCθ in T cells and PKCβ in B cells, are responsible for phosphorylating CARMA1 and causing a shift from an autoinhibited configuration into the active configuration, which binds to membrane lipid rafts, oligomerizes, and recruits Bcl10, which in turn recruits MALT1 and TRAF2 and/or TRAF6. TRAF family members are required components for the activation of NF-κB by most and possibly all receptors. TRAFs interact with the IKK complex and with the protein kinase that is believed to directly activate it, TAK1. These interactions, promoted by the TAK1-associated proteins TAB1, TAB2, and TAB3, transmit the information of Bcl10 and MALT1 oligomerization into IKK activation. NF-κB activity is also modulated by phosphorylations of the p50 and p65 subunits, but the exact details are not well understood.

RAS ACTIVATION IN T AND B CELLS

Ras proteins have been implicated in a wide variety of growth and differentiation responses in many cell types. The activation of Ras proteins following antigen receptor stimulation has similarly been shown to be a critical event in lymphocyte development and activation. The Ras proteins are a small family of 21 kDa GTP-binding proteins with GTPase activity. The activation state of Ras is determined by the form of guanine nucleotide bound to it; GTP-bound Ras is active and GDP-bound Ras is inactive. GDP-bound Ras becomes activated by interacting with guanine nucleotide exchange proteins (GEFs), which cause it to release GDP, allowing it to bind GTP (Fig. 6.6). Conversely, GTP-bound Ras is inactivated by hydrolyzing the GTP to form GDP and PO_4, a reaction stimulated by GTPase activating proteins (GAPs). Thus, the activation of Ras could be a consequence of GEF stimulation, GAP inhibition, or a combination of both. The activation of Ras can lead to a variety of downstream events, including (1) the activation of the Erk/MAPK pathway; (2) the activation of PI 3-kinase; and (3) the activation of other small-molecular-weight, GTP-binding proteins such as Rac and CDC42 (McCormick and Wittinghofer, 1996).

Initial studies of growth factor receptor protein tyrosine kinases (PTKs) in Drosophila, *C. elegans*, and mammalian fibroblasts identified an adaptor molecule called Drk, Sem 5, or Grb2, respectively, in the different species, that interacts with both the stimulated receptors and with a guanine nucleotide exchange factor called SOS (son of sevenless) to activate Ras. Subsequently, in some systems another adaptor, called Shc, was found to function in linking Grb2/SOS to the activated receptor PTKs. Genetic and biochemical studies indicate that Grb2/SOS proteins couple the stimulated PTK receptors to the activation of Ras. SOS action on Ras is

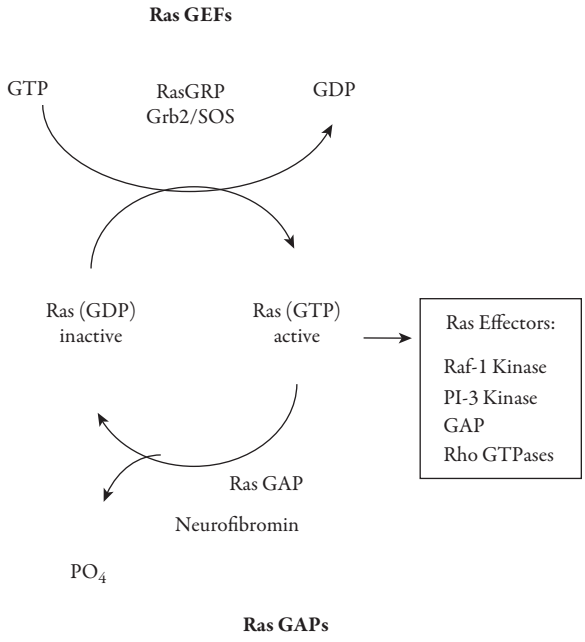


Figure 6.6 ***Regulation of Ras activation and its effector functions.*** Ras cycles between an active form in which GTP is bound and an inactive form in which GDP is bound. Conversion to the active form involves dissociation of bound GDP, catalyzed by Ras GEFs (guanine nucleotide exchange factors), followed by binding of GTP, whereas conversion to the inactive form involves hydrolysis of GTP, stimulated by RasGAPs (GTPase activating proteins), to yield free phosphate, which dissociates from Ras, and GDP, which stays bound to the now-inactive Ras.

regulated, in part, by intracellular localization: receptor signaling induces a translocation of Grb2/SOS from the cytosol to the plasma membrane by virtue of the binding of Grb2 to tyrosine phosphorylated sites on the receptor itself or on adaptor proteins such as Shc. Shc phosphorylation and interaction with Grb2/SOS has been observed after TCR or BCR stimulation (Crowley et al., 1996; Ravichandran et al., 1993; Saxton et al., 1994). Conditional deletion of the *Shc1* gene in developing thymocytes indicates that the majority of Erk activation at the TCR β-selection checkpoint is dependent upon ShcA (Trampont et al., 2006), although the importance of Shc for activation of Ras and ERK in mature lymphocytes is unclear.

Activation of GEF function is probably the primary mechanism responsible for the positive activation of Ras by the TCR and BCR (Ravichandran et al., 1993). In T cells, the Grb2/SOS complex associates with the TCR-induced phosphorylated LAT, which would serve to translocate these complexes to the membrane and can stimulate Ras activation (Wange, 2000). However, optimal Ras activation in T and B cells appears to involve another family of Ras GEFs that are activated downstream of PLCγ1 or 2 activation and activate Ras in concert with SOS proteins (Dower et al., 2000; Ebinu et al., 2000). RasGRP proteins have calcium-binding EF hands and, more importantly, a C2/DAG-binding domain. Thus, the activity of PLCγ1 may be directly translated into Ras activation through the recruitment of RasGRP. In addition, PKC enzymes, which are also activated by DAG together with calcium in the case of the classical PKC isoforms, play a role in activating RasGRP GEF function by phosphorylating RasGRP.

Regulation of Ras activation through RasGAPs also occurs to some extent. For example, RasGAP is tyrosine phosphorylated following BCR stimulation and to a lesser extent following TCR stimulation (Gold et al., 1993). In B cells, it is recruited to the membrane via the negative coreceptor FcγRIIB, SHIP, and the adaptor protein $p62^{dok}$ (Tamir et al., 2000; Yamanashi et al., 2000), so it appears to function to limit Ras activation at least in this context.

Why should lymphocyte antigen receptors utilize two families of Ras GEF, SOS proteins and RasGRP proteins, for the activation of Ras? Recent studies suggest very dynamic and synergistic functional interactions between SOS and RasGRP proteins (Das et al., 2009). SOS proteins can interact with Ras via two sites. One binds to the GDP-bound form of Ras and induces exchange with GTP. The other site, the so-called allosteric site, binds preferentially to the active GTP-bound form of Ras proteins, and this interaction greatly stimulates the GEF activity of the other Ras-binding site of SOS (Boykevisch et al., 2006). Thus, there is the potential for positive feedback influencing SOS activity. Recent studies suggest that RasGRP may facilitate the activation of SOS activity following TCR engagement by providing the initial low levels of RasGTP that can boost the activity of SOS, which is also recruited to the membrane (Das et al., 2009). Moreover, the coordinated activation of both of these two GEFs exhibits hysteresis, or a type of biochemical memory, which prolongs Ras activation even after a stimulus is removed. This memory can facilitate Ras activation in lymphocytes in the setting of transient cell–cell interactions in secondary lymphoid organs.

As listed above, there are several reported downstream effectors of Ras (Alberola-Ila and Hernandez-Hoyos, 2003). The effectors responsible for Ras function in different systems are not fully established and no single effector can usually substitute for the activated form of Ras in experimental models. The best-characterized Ras effector is the Raf-1 kinase (Morrison and Cutler, 1997). GTP-bound Ras interacts directly with the serine/threonine kinase Raf-1, translocating it to the membrane. The translocation of Raf-1 to the membrane can increase its activity, although other mechanisms have been implicated in Raf activation, including its tyrosine phosphorylation and transphosphorylation by dimerization. The translocation to the membrane by Ras may simply concentrate Raf-1 there and allow it to dimerize and transphosphorylate. In any case, Raf-1 is activated in T and B cells in response to TCR and BCR stimulation or phorbol ester stimulation, corresponding to signals activating Ras (Genot and Cantrell, 2000). The activation of Raf-1 leads to its direct interaction and activation of a dual specific tyrosine/serine/threonine kinase, MEK-1, that in turn activates the Erk1/Erk2 MAP kinases. MAP kinases have numerous functions, including the phosphorylation and activation of transcription factors such as serum response factor, which can regulate c-fos and Egr-1 transcription (Raman et al., 2007). Thus, this antigen receptor-regulated cascade of events ending in MAP kinase

activation can function to regulate transcriptional events leading to lymphocyte differentiation and activation.

Ras plays a critical role in lymphocyte development and activation. For example, expression of activated Ras as a transgene can promote early T-cell developmental transitions in recombinase activating gene (RAG)-deficient mice, which cannot express the pre-TCR (Swat et al., 1996). Similarly, blockade of the Ras pathway with inactivating mutants can interrupt normal thymic development (Alberola-Ila and Hernandez-Hoyos, 2003). The magnitude of Ras activation may be very critical for determining whether TCR peptide/MHC molecule interactions lead to positive or negative selection in the thymus. Negative selection has been correlated with strong but transient Erk activation, whereas positive selection requires a lower but sustained elevation of Erk activity (McNeil et al., 2005). In addition, the localization of activated Erk within the developing thymocyte also differed between thymocytes undergoing negative selection and those undergoing positive selection (Daniels et al., 2006). Recent modeling studies suggest mechanisms whereby a sharp threshold of TCR peptide/MHC molecule affinity can result from the extent of LAT phosphorylation and properties of the Grb2/SOS and RasGRP GEFs (Prasad et al., 2009), which may contribute to the sharp affinity cutoff between positive selection and negative selection in the thymus (Naeher et al., 2007).

Ras activation is also critical for T-cell activation. Dominant negative mutants of Ras can partially block IL-2 gene activation in T cells and completely block NFAT-based transcription (Genot and Cantrell, 2000). This is probably explained by the involvement of Ras in regulating the AP-1 components involved. Conversely, an activated form of Ras, which cannot hydrolyze GTP, can synergize with calcium ionophores to induce NFAT-directed transcription.

Similarly, Ras plays important roles in B-cell development and activation. Specific inhibition of the Erk MAPK by using pharmacological inhibitors of MEK1 and MEK2 blocked BCR-induced proliferation of mature B cells but did not block apoptotic responses of immature B cells and an immature B cell line, indicating that Erk MAPK mediates some effects of BCR signaling but not others (Richards et al., 2001). BCR-induced transcription of the *egr*-1 early response gene is mediated by Ras (McMahon and Monroe, 1995). The Egr-1 promoter contains five serum response elements (SREs), sites of action of SRF, which is activated downstream of Ras, Raf, and Erk. Egr1 appears to mediate BCR-induced upregulation of ICAM-1 and CD44 expression, molecules that are important for B-cell adhesion to other cells (Maltzman, Carman et al., 1996; Maltzman, Carmen et al., 1996).

VAV PHOSPHORYLATION AND FUNCTION IN T AND B CELLS

Among the many TCR- and BCR-induced tyrosine phosphoproteins is the proto-oncogene Vav (Margolis et al., 1992). The original cellular Vav is now called Vav-1, as mammals additionally express two related molecules. Vav-1 is expressed exclusively in hematopoietic cells and trophoblasts, whereas Vav-2 and Vav-3 are expressed more widely (Swat and Fujikawa, 2005). Vav-1 contains multiple distinct domains, including a leucine-rich domain, a domain related to GEF domains of Rho family GTPases (Dbl-homology domains), a pleckstrin homology (PH) domain, a zinc finger domain, two SH3 domains, and an SH2 domain. Deletion of the N-terminal 65 residues of Vav leads to a protein with transforming ability in NIH 3T3 fibroblasts (Katzav et al., 1991).

Vav-1 appears to be a direct substrate of the TCR- and BCR-dependent Src family kinases and Syk family PTKs (Swat and Fujikawa, 2005). Phosphorylation of tyrosines near its N-terminus seems to play a role in activating the GEF function of the Dbl-homology domain. Its SH2 domain has a consensus binding specificity for the sequence adjacent to Y315 (YESP) in ZAP-70 (Songyang et al., 1994). Both the binding of the SH2 domain of Vav-1 and the tyrosine phosphorylation of Vav-1 depend on this site in ZAP-70 (Wu et al., 1997). Moreover, the functional activity of ZAP-70 is reduced by mutation of this site in a reconstitution system where NFAT activity is assessed. These results suggest that the interaction of ZAP-70 with Vav is an important event in antigen receptor signal transduction.

The importance of Vav in TCR and BCR signaling is underscored by the phenotype of mice lacking one or more Vav family members (DeFranco, 2001). The Vav-1-deficient mice show defects in thymic cellularity, thymocyte positive and negative selection, peripheral T-cell numbers, and peripheral T-cell responses (Swat and Fujikawa, 2005; Tybulewicz et al., 2003). While the deficiency of Vav-1 does not have any serious defects in the B-cell compartment other than loss of the B-1 subtype of B cells, evidence from genetic knockouts of the Vav-2 gene alone or in combination with Vav-1 deficiency shows that Vav-1 and Vav-2 have both unique and redundant roles in BCR-mediated signaling (Doody et al., 2001; Tedford et al., 2001). The development of T and B cells in Vav-2-deficient mice is largely unaffected; however, these mice show a decrease in antibody responses to T-independent type 2 antigens and also a partial defect in T-dependent IgG production. Suboptimal stimulation of the BCR revealed defects in BCR responses such as calcium flux and proliferation in these mice. Vav-1 and Vav-2 double knockout mice, by contrast, had impaired B-cell development and greatly diminished BCR responses, similar to the substantial effects of the Vav-1 knockout on T cells. Deletion of all three Vav proteins leads to a severe impairment in T- and B-cell development (Swat and Fujikawa, 2005).

Considerable evidence suggests that the GEF-dependent function of Vav regulates Rho, Rac, or Cdc 42 GTP-binding proteins (Bustelo, 2001; Swat and Fujikawa, 2005). The primary role of these Ras-like small-molecular-weight GTPases is to orchestrate changes in the actin cytoskeleton. Such changes are likely important for effective interactions of T cells with antigen-presenting cells (Dustin, 2009) or of B cells with membrane-bound antigen (Harwood and Batista, 2008). In one model system used to probe Vav-1 function, overexpression of Vav in Jurkat T cells led to potentiation of TCR-mediated IL-2- and NFAT-regulated reporter constructs (Wu et al., 1995). This functional activity, which depends on TCR-specific signals, is also dependent on Ras,

Lck, and calcineurin function. This suggests that Vav may function very proximally within the TCR pathway or in an alternate signaling pathway that, together with Ras- and calcineurin-regulated pathways, contributes to IL-2 gene expression. This is most likely to involve increased Rac and Cdc42 activity, as evidenced by studies of Vav-deficient cells. The most prominent signaling defect in these cells is a failure of actin polymerization and antigen receptor clustering or cap formation (Fischer et al., 1998). These effects suggest that the regulation of Rac and Cdc42 by Vav GEF function is a critical event in TCR signal transduction.

Surprisingly, biochemical defects in TCR signaling in Vav-1-deficient T cells include defects in phospholipase C tyrosine phosphorylation and Ca^{2+} mobilization (Tybulewicz et al., 2003). This global effect of Vav deficiency suggests that Vav may have roles in T-cell activation beyond Rac or Cdc42 activation. It is possible that Vav plays an important role in the formation or stability of TCR-induced multicomponent signaling complexes (signalosomes). Vav interacts with the TCR- and BCR-induced adaptors, SLP-76 (Wu et al., 1996), and BLNK (Fu et al., 1998). The Vav SH2 domain is required for these interactions. As has been seen with Vav, overexpression of SLP-76 markedly potentiates TCR-mediated IL-2 promoter and NFAT-driven gene expression (Motto et al., 1996). Furthermore, overexpression of both Vav and SLP-76 synergistically induced basal and TCR-stimulated NFAT activity (Wu et al., 1996). These results suggest that a signaling complex containing Vav and SLP-76 plays an important role in T-lymphocyte activation. At least part of this function may be GEF-independent and may relate to an adaptor function of Vav (Kuhne et al., 2000). Hence, the defects in MAPK activation and in PI 3-kinase activation that have also been observed in Vav-1-deficient T cells may relate to such adaptor functions.

In B cells, all three Vav isoforms seem to participate in BCR signaling (Kurosaki and Hikida, 2009). Syk contains a sequence homologous to the Vav-1 binding site of ZAP-70, and in addition, the cytoplasmic domain of CD19 has a binding site for Vav. Recent studies suggest that while CD19 appears to be dispensible for early BCR signaling in response to soluble ligands, it is essential for robust signaling in response to membrane-bound ligands, which are likely to be important in many circumstances in vivo (Harwood and Batista, 2008). Genetic experiments in mice and in DT-40 chicken B cells demonstrate that CD19, Vav, and Rac2 are all essential for actin-based spreading of B cells following contact with antigen on a cell surface. This spreading allows the B cell to sense the presence of antigen with much greater sensitivity and thereby enable signaling and also antigen uptake for presentation to helper T cells. Thus, as is the case in T cells, Vav is likely to play a critical role in B-cell activation via its ability to promote actin polymerization to support cell–cell contact.

ANTIGEN RECEPTOR-MEDIATED ACTIVATION OF INTEGRINS

A key function of antigen receptors is to regulate the adhesion of T cells and B cells to cells presenting antigen to them, and this is largely accomplished via regulation of the adhesive properties of integrins (Evans et al., 2009). Integrins are a family of αβ heterodimeric transmembrane proteins that on their intracellular side bind to actin cytoskeleton components and on their extracellular side bind either to cell-surface ligands on other cells, or less importantly in the case of lymphocytes, to extracellular matrix ligands. Hence, they "integrate" cell binding to their environmental cues with binding to the cytoskeleton. Their affinity is regulated in two major ways: by their conformation and by their lateral mobility as controlled by the actin cytoskeleton. In their low-affinity conformation, integrin extracellular domains are bent sharply in the middle, with the end of the molecule pointing back toward the cell expressing the integrin. Signaling events induced by the TCR or BCR signaling, or alternatively by chemokine receptors, induce a straightening of the molecule so that the end is now farther away from the cell and in a better position to bind its ligand. Integrins appear to exhibit two higher-affinity states, referred to as an intermediate-affinity state and a high-affinity state, but what controls the shift between intermediate-affinity and high-affinity states is not well understood. So, here we focus on the shift between the low-affinity and one or the other of the higher-affinity states. In addition to this affinity regulation, integrins generally are fixed to the cytoskeleton, and this limits their ability to bind to ligands that may also be fixed in position (in the extracellular matrix) or at least somewhat limited in their mobility (on the surface of antigen-presenting cells or endothelial cells). TCR or BCR signaling induces dramatic remodeling of the actin cytoskeleton, and this causes a transient release of integrins from being tethered to the actin network. Once these molecules bind to their ligands using their newly acquired higher-affinity conformation, they again become fixed to the cytoskeleton and mediate strong adhesion to the antigen-presenting cell, at least temporarily. This rapid and dramatic alteration in their contribution to cell-to-cell or cell-to-matrix adhesion makes integrins well suited to supporting the function of T cells and B cells. In the integrin field, the ability of other receptors (TCR and BCR in this case) to promote integrin-based adhesion is referred to as "inside-out" signaling, whereas the ability of integrins that have bound their adhesive ligands to signal that fact to the cell and thereby alter cell behavior (survival, activation, etc.) is referred to as "outside-in" signaling.

Both the TCR and the BCR mediate powerful inside-out signaling to induce integrin adhesion. Current understanding of the molecular mechanisms is moderately well advanced, but some questions remain and it is unclear at this time how directly analogous are the molecular mechanisms in T cells and B cells. In T cells, genetic evidence has demonstrated that TCR-induced integrin adhesion depends on the phospholipase Cγ1 signaling pathway, including activation of PKCθ, and also protein–protein interactions emanating from SLP-76 (Baker and Koretzky, 2008). Upon TCR engagement, SLP-76 associates with an adaptor called ADAP (adhesion and degranulation promoting adaptor protein), which in turn associates with an adaptor called SKAP-55 (Src kinase-associated phosphoprotein of 55kDa). SKAP-55 in turn associates with

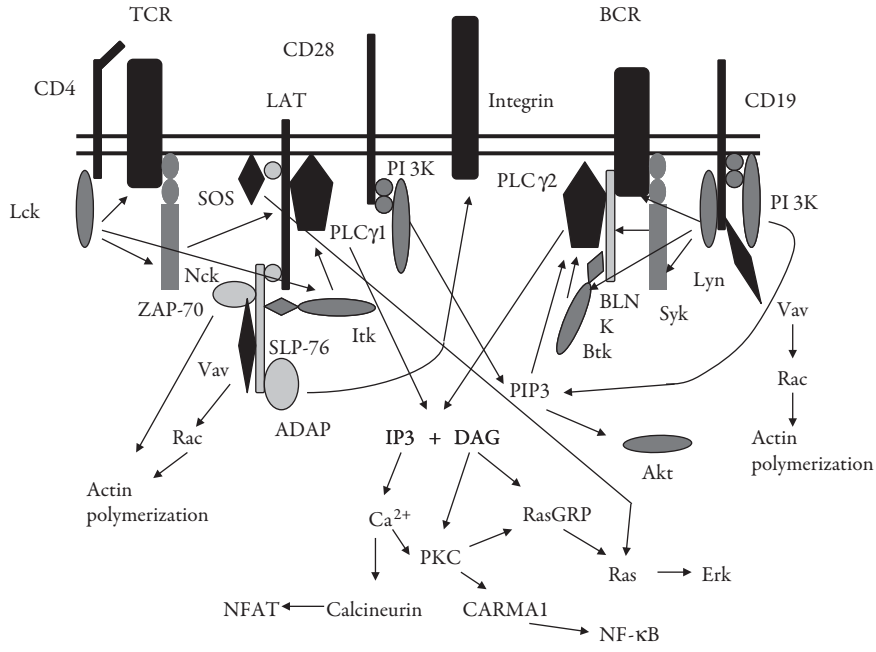


Figure 6.7 *Antigen receptor signaling induces formation of signaling complexes.* See text for details. Not all known interactions could be shown for sake of clarity.

RIAM (Rap1-interacting adhesion molecule). This complex is thought to localize and activate the small-molecular-weight GTP-binding protein Rap1, which appears to be a key activator of integrin affinity, perhaps via its putative effector RapL. In addition, TCR signaling promotes integrin mobility in the membrane, possibly via PKCθ, but how this occurs is not known.

In the case of B cells, the mechanism by which antigen receptor signaling promotes integrin adhesion is less well worked out, but a central role for Rap1 is well established (Arana et al., 2008; Lin et al., 2008; McLeod et al., 2004). Mature B cells do not express ADAP or SKAP-55 but do express a molecule similar to the latter, called SKAP-HOM, and B-cell integrin adhesion is dependent on this SKAP isoform (Togni et al., 2005). Upstream signaling events that appear to be important include PI 3-kinase activation, Vav1/2 activation, and Rac2 (Arana et al., 2008). How integrin activation is localized is not yet clear in B cells, although CD19 may play a critical role.

The major signaling pathways of TCR and BCR signaling are summarized in Figure 6.7.

COMPARTMENTALIZATION OF T AND B ANTIGEN RECEPTOR SIGNALING

Signaling via the antigen receptors and recruitment of various signaling molecules into a "signalosome" is increasingly being viewed in the cell biological context. Recent studies indicate that the interaction of lymphocytes with antigen bound to cell surfaces results in intriguing cell biological phenomena at the point of cell–cell contact, referred to as an "immunological synapse" (Dustin, 2009).

A ROLE FOR PLASMA MEMBRANE MICRODOMAINS IN SIGNAL TRANSDUCTION?

A controversial area in the signaling by antigen receptors has been the role of microdomains in the plasma membrane called "lipid rafts," or more recently and more accurately "membrane rafts" (Dykstra et al., 2003; Gupta and DeFranco, 2007; Shaw, 2006). These subdomains of the plasma membrane are rich in cholesterol, sphingomyelin, and glycosphingolipids with saturated fatty acyl chains, which gives them a less fluid, more structured character referred to as "liquid ordered." These subdomains are enriched in a subset of membrane proteins, including those with particular lipid modifications, such as glycosylphosphatidylinositol (GPI)-anchored proteins, palmitoylated proteins such as members of the Src kinase family, heterotrimeric G proteins, some adaptors such as LAT, and isoprenylated proteins, including Ras family small G proteins. In resting cells, membrane rafts are small (5 to 200 nm in diameter), highly dynamic structures, but upon activation, these microdomains coalesce into structures large enough to be visible by fluorescence microscopy (Viola and Gupta, 2007). Controversy surrounding their role in antigen receptor signaling largely stems from the methods that were commonly used in early studies, namely cholesterol depletion and biochemical isolation by differential detergent solubility (Shaw, 2006). Cholesterol depletion of cells with agents such as methyl-β-cyclodextran causes the remaining membrane-raft components to dissociate and become incorporated into the bulk membranes of the cell. Clearly many membrane functions are perturbed by cholesterol depletion, not just membrane-raft association, so interpreting the results of cholesterol-depletion experiments is not straightforward.

Putative membrane rafts can be isolated for biochemical analysis by taking advantage of their resistance to extraction

by nonionic detergents at low temperature, a feature that is believed to result from the more stable packing of the lipids in these microdomains. While there is some concern that this procedure could create structures that do not exist in the cellular membranes, a greater uncertainty for interpreting data from this approach is that the protein composition of these "detergent-resistant membranes" (DRMs) varies depending on the concentration of detergent used, so whether or not some proteins are components of membrane rafts is not clear. In general, antigen receptors are found to be largely absent from DRM preparations prior to antigen engagement but present in such preparation soon after antigenic stimulation is initiated (Dykstra et al., 2003). This change in localization of antigen receptors upon engagement has been verified by fluorescence microscopy in living B cells by using fluorescence resonance energy transfer (FRET), which showed a rapid association of the BCR with membrane-raft structures identified by the presence of a fusion protein between the membrane-raft targeting N-terminal sequence of Lyn fused to GFP (Sohn et al., 2006, 2008). Moreover, lipid rafts appear to be home to a number of proteins important for antigen receptor signaling, including the co-receptors CD4 and CD8, Src family kinases Lck, Lyn and Fyn, and transmembrane molecules that participate in signaling such as LAT and PAG-1 (Dykstra et al., 2003). In addition, CARMA1, which is essential for antigen receptor activation of NF-κB, is induced to associate with membrane rafts upon stimulation (Rawlings et al., 2006).

Despite these suggestive observations, direct evidence for the role of membrane rafts in antigen receptor signaling is scant. Efforts were made to test the role of localization of LAT to membrane rafts for supporting TCR signaling by mutation of the two LAT juxtamembrane Cys residues. These residues are palmitoylated, and these modifications are likely to target LAT to membrane rafts. Mutation of these residues abolished the ability of the mutated LAT to participate in TCR signaling. It is now apparent, however, that this experiment did not test the role of localization of LAT to membrane rafts for its participation in TCR signaling because the mutated LAT no longer trafficks to the plasma membrane (Hundt et al., 2009; Zhu et al., 2005). Morover, LAT does not need to be targeted to membrane rafts to participate in TCR signaling effectively, as determined using a chimeric molecule containing the cytoplasmic domain of LAT and targeting signals that take it to the plasma membrane but not to membrane rafts. Nonetheless, the approach of altering the localization of key antigen receptor signaling components from membrane rafts to the non-raft membrane is promising and likely could provide additional insights into the role of membrane-raft compartimentalization.

Membrane rafts are linked to the actin cytoskeleton, and this association appears to maintain these structures in a dispersed state. This linkage depends in large part on the ERM proteins (ezrin, radixin, moiesin), which are cytosolic proteins that in the active configuration bind to a variety of plasma membrane proteins and also to the actin cytoskeleton, linking the two. Stimulation of the BCR or the TCR results in a rapid dephosphorylation of ezrin, converting it to the inactive confirmation, with the result that there is dissociation of ezrin and a decreased linkage of the plasma membrane to the actin cytoskeleton. This release was shown to improve the ability of T cells to interact with antigen-presenting cells, probably by reducing the rigidity of the plasma membrane (Faure et al., 2004). In B cells, the release of ezrin was found to permit reorganization of membrane rafts within the plasma membrane, allowing for the coalescence of small lipid rafts into larger structures (Gupta et al., 2006). One interesting hypothesis is that these larger rafts now contain at least one of each required signaling component and thereby can efficiently support signaling.

Interestingly, the developmental stage of B and T cells influences the ability of the BCR or TCR to enter membrane rafts upon engagement. Whereas antigen receptors translocate to membrane rafts in mature lymphocytes, they fail to do this in immature B cells or T cells (Viola and Gupta, 2007). In the case of immature B cells, this was shown to be due to a lower level of cholesterol in the plasma membrane of immature B cells relative to mature B cells (Dykstra et al., 2003). Moreover, supplementation of the membranes of immature B cells with cholesterol restored the ligand-induced association of the BCR with membrane rafts (Karnell et al., 2005). These observations indicate that membrane rafts are unlikely to play an essential role in antigen receptor signaling. It is possible that they serve to amplify signaling or even may be required for a subset of signaling reactions. In agreement with the later idea, immature B cells were poor at activating Rac1 in response to BCR cross-linking, whereas cholesterol-supplemented immature B cells activated Rac1 similarly to mature B cells (Brezski and Monroe, 2007). An alternative and not mutually exclusive possibility is that the dispersion of signaling components in small membrane rafts inhibits signaling at low levels of antigen receptor engagement and creates a greater threshold for signaling in mature B cells compared to immature B cells, consistent with observations in the literature (Gross et al., 2009). Additional experiments will be needed to address these possibilities.

ORGANIZATION OF ANTIGEN RECEPTORS IN THE IMMUNOLOGICAL SYNAPSE

Efficient T-cell activation requires sustained interaction between the TCR and MHC/peptide complex. Recent imaging studies of regions of contact between T cells and antigen-presenting cells have described the following sequence of events occurring at the plasma membrane: T-cell polarization, initial adhesion, TCR microcluster formation, and formation of a highly organized immunological synapse. T cells use the leading edge of the cell, the lamellapodia, to contact the surface of the antigen-presenting cell and test for the presence of antigen. Once TCRs in the lamellapodia become engaged by their cognate peptide/MHC complex, they organize into small aggregates called "microclusters," which are visible by using a high-resolution form of fluorescence microscopy called total internal reflection fluorescence (TIRF) microscopy, together with an artificial lipid bilayer containing peptide/MHC complexes and ligands for integrins (Dustin, 2009). These TCR microclusters are highly active in signaling

and are connected to the actin cytoskeleton, which sweeps them to the center of the contact region. Within minutes of a stable T cell–APC interaction, the molecules in the contact zone organize themselves into a bull's-eye arrangement of molecules with the TCR and MHC/peptide complexes clustering in the center (central supramolecular activation clusters, c-SMACs) and the adhesion molecules, LFA-1 and ICAM-1, forming a ring around the periphery (p-SMACs).

The functional significance of the c-SMAC/p-SMAC organization of the immunological synapse has been a matter of great debate. When a strong antigenic stimulus is provided to the T cell, microclusters outside of the p-SMAC exhibit strong signaling, whereas signaling in the c-SMAC area is attenuated (Varma, 2006), arguing that inhibitory mechanisms limiting or terminating signaling are favored in the c-SMAC in this circumstance. In agreement with this interpretation, the use of a patterned artificial membrane that allows formation of TCR microclusters but does not allow them to be swept into the c-SMAC results in enhanced TCR signaling compared to a nonpatterned bilayer (Mossman et al., 2005). In contrast, when a weak antigenic stimulus is provided to the T cell, more TCR signaling is evident within the c-SMAC region than in the peripheral regions (Cemerski et al., 2007, 2008), suggesting that attenuation of signaling in the c-SMAC is dependent on the level of signaling. Mathematical modeling based on these observations suggests that for this reason the c-SMAC/p-SMAC organization of the immunological synapse serves to broaden the responsiveness of T cells to allow them to respond to weaker antigens as well as stronger antigens.

Co-stimulation via CD28 also affects aspects of the immunological synapse. When CD80 and/or CD86 is expressed on the antigen-presenting cell, then CD28 is observed to be present in the early signaling TCR microclusters, and it is brought to a region adjacent to but outside the c-SMAC region containing the TCR (Yokosuka, Kobayashi et al., 2008). The participation of CD28 seems to provide qualitatively unique aspects to the immunological synapse, including efficient recruitment of PKCθ (Altman and Villalba, 2003) and also efficient recruitment of membrane-raft microdomains (Viola and Gupta, 2007). The latter function is mediated by the actin filament cross-linking protein filamin A, which associates with the cytoplasmic domain of CD28 in the c-SMAC and promotes association with membrane rafts via the actin cytoskeleton (Tavano et al., 2006).

The immunological synapse is usually thought to be a property of a T cell interacting with an antigen-presenting cell, but a similar cell biology is seen in the interaction between B cells and antigen-bearing follicular dendritic cells (FDCs) or antigen- and integrin ligand-bearing artificial membrane bilayers (Harwood and Batista, 2008). B cells contacting a membrane-bound antigen initiate a spreading response that extends the area of membrane contact between the B cell and the bilayer. Microclusters of signaling BCRs are rapidly formed, highly analogous to what is seen in T cells. These microclusters are swept into a c-SMAC area of contact and the B cell contracts its membrane, retaining contact with the antigen-bearing membrane in the central area. As in T cells, the antigen receptor-rich cSMAC is surrounded by a ring of LFA-1. This sequence of events is dependent on BCR signaling reactions, including those downstream of Vav and PLC-γ2 (Weber et al., 2008), and facilitates uptake of the bound antigen, allowing the B cell to then process the antigen and load it onto its class II MHC molecules for presentation to helper T cells.

The demonstration that B cells and T cells interacting with membrane-bound antigen exhibit nearly identical cell biological organization of their antigen receptors and integrins suggests that this behavior reflects the biophysical and cell biological nature of the events. For example, the molecular sizes of integrin-ligand extracellular domains and of TCR-MHC extracellular domains are different enough that it has been proposed that the size differences provide a force for segregating these molecules into distinct zones (Choudhuri and van der Merwe, 2007). On top of this biophysical force, the actin cytoskeleton together with myosin IIA (Ilani et al., 2009) actively moves TCR microclusters to the center of the contact region, which may be responsible for forcing integrins to a ring surrounding the c-SMAC.

CONCLUSIONS

Although T and B cells use distinct receptors to recognize completely different forms of antigens, the signal transduction machinery regulated by their antigen receptors shows many similarities. Differences may reflect the different form or context in which antigen is recognized. The signaling machinery includes lymphocyte-specific components that interact with signaling components that are more ubiquitously expressed and used in a wide variety of cellular systems. Lymphocyte function may be impaired by interruption of any of the critical components in these signaling cascades. However, discrete immune deficiency states are likely to result from alterations in the critical known and unknown components that are lymphoid-specific.

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7.

LYMPHOID ORGAN DEVELOPMENT, CELL TRAFFICKING, AND LYMPHOCYTE RESPONSES

Sirpa Jalkanen and Marko Salmi

A productive interaction between a lymphocyte and its cognate antigen is required to trigger an adequate immune response. Thus, it is not only the defects in the production and maturation of lymphocytes or in the activation machinery that can result in immunodeficiencies, but the same outcome can be seen if otherwise normal cells do not migrate properly within the body in search of foreign antigens. The best-known examples of this class of diseases are leukocyte adhesion deficiencies (LAD, see Chapter 38). The impact of proper lymphocyte recirculation between blood and different tissues and within the tissue stroma for eliciting an immune response already becomes evident by looking at the physical dimensions of our immune system.

Humans have approximately 10^{12} lymphocytes, each of which normally expresses receptors for only one antigen. Thus, for most antigens only some 100 to 1,000 specific lymphocytes reside anywhere in the body. Moreover, at any moment only about 1 percent of these lymphocytes are in blood circulation, whereas the rest are in tissues. The freely mobile cells in the blood travel at high velocity for their size. Even in small-caliber venules, where the extravasation takes place, an 8-μm-diameter cell moves about 500 μm/sec in the blood flow. Moreover, the total length of the circulatory tree has been estimated to exceed 100,000 km in an adult. These constraints make it very challenging for a specific cell to leave the blood at a desired site. At the same time, enormously large body surfaces are exposed to antigen invasion. The total area of the principal entry ports for antigens is about 2 m^2 in the skin, 400 m^2 in the gut, and 100 m^2 in the respiratory system. The antigens, on the other hand, can be minute particles such as viruses or short foreign peptides. Thus it is clear that a highly efficient, adaptive immune response cannot rely on chance for random contact between the antigen and a specific lymphocyte anywhere in the body.

An elaborate system of lymphocyte recirculation has evolved to address these challenges (Butcher and Picker, 1996; Salmi and Jalkanen, 1997; Springer, 1994). Different functions of the immune system are compartmentalized to specific organs and tissues, and these units are then interconnected by lymphocyte trafficking via trees of vascular and lymphatic vessels (Fig. 7.1). Thus, primary lymphoid organs are specialized for the production of huge numbers of lymphocytes. The specificity of a lymphocyte is also determined in these organs. The mature but antigenically inexperienced cells are then released into the circulation. These naïve cells extravasate from the blood into secondary lymphoid organs (such as peripheral lymph nodes, Peyer's patches of gut). Simultaneously, antigens are concentrated into these very same organs from large epithelial surfaces (via an afferent lymphatic system to peripheral lymph nodes and via M cells into Peyer's patches). The secondary lymphoid organs are thus designed to collect and concentrate lymphocytes on the one hand and antigens on the other and to provide an optimal microenvironment for launching a productive immune response. Finally, the effector lymphocytes leave the secondary lymphoid organs, equipped with a capacity to migrate to the peripheral sites of inflammation. All the rest of the organs and tissues in the body can be regarded as tertiary immune tissues, which harbor significant numbers of lymphocytes only during an active immune response.

LYMPHOCYTE MIGRATION TO PRIMARY LYMPHATIC ORGANS

Lymphocyte trafficking first takes place during early ontogeny, when lymphoid precursor cells populate the lymphocyte-forming organs (Cumano and Godin, 2007; Ueno and

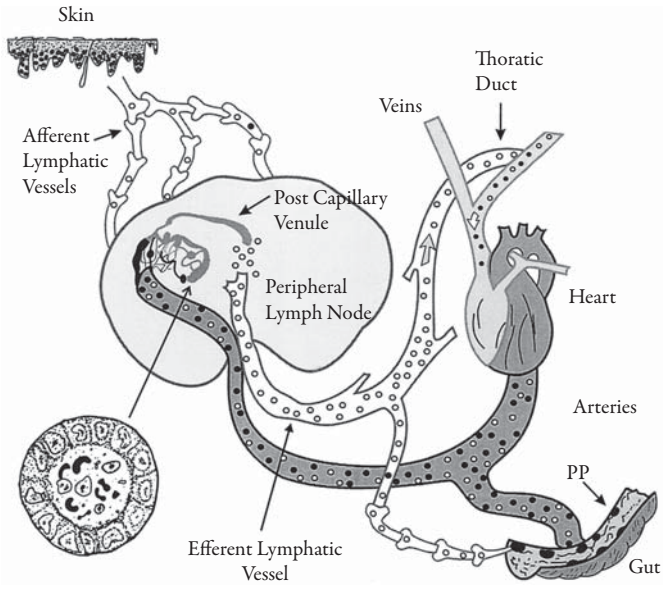

Figure 7.1 Lymphocytes recirculate between the blood and lymphoid organs. Most lymphocytes enter the peripheral lymph nodes or organized lymphoid tissues of the gut (Peyer's patches [PP] and appendix) via high endothelial venules. A low-level, continuous migration of lymphocytes from vascular beds such as skin to lymph nodes takes place via afferent lymphatics. The incoming lymphocytes migrate through the tissue parenchyma, enter the lymphatics, and are then carried within the efferent lymphatics back to the systemic blood circulation. Most of the venous circulation has been omitted for clarity. (Reproduced with permission from Salmi and Jalkanen, 1997.)

Weissman, 2007). Initially, precursor cells from the yolk sac and/or aorta–gonad–mesonephros area migrate to the fetal liver. Later, the spleen and bone marrow become the main targets for these precursor cells. After this stage the fate of B and T lymphocytes differs. B lymphocytes undergo their entire development in the cavity of bone marrow in contact with stromal cells. T-cell precursors, by contrast, move further on to the thymus. They enter the thymus through cortical vessels and gradually migrate toward the medulla during the maturation processes. Finally, immunocompetent but naïve B and T lymphocytes join the circulating pool of lymphocytes in the blood. Although these early migratory events are crucial for the development of the immune system, many of the molecular determinants involved in the trafficking remain to be elucidated.

Development of the lymphoid organs and their population of lymphocytes is regulated in a highly time-dependent manner (Bailey and Weiss, 1975; Bofill et al., 1985; Campana et al., 1989; Spencer et al., 1986; Vondenhoff et al., 2007). In humans, the first CD3-positive thymocytes are found by week 10 of gestation. Anlage of secondary lymphoid organs have already started to develop earlier. Peripheral lymph nodes developing from a lymphatic plexus become recognizable around week 10, but development of high endothelial venule (HEV)-like vessels (the specialized vessels for lymphocyte recruitment, see below) and accumulation of early lymphocytes in these nodes can be seen only in 15- to 18-week-old fetuses. In gut, mucosal villi appear around weeks 9 to 10 of gestation. By week 14, clusters of lymphocytes can be detected in the lamina propria, and by 19 weeks well-organized Peyer's patches appear. The 12-week-old fetal spleen contains few lymphocytes, but at 14 weeks T and B cells are scattered throughout the organ. The early seeding of lymphocytes takes place in the absence of any external antigenic stimulation.

It is intriguing that the endothelial adhesion molecules, which govern lymphocyte trafficking in the adult, are expressed in a functional form very early during human development but in a remarkably different manner in terms of tissue specificity (Salmi et al., 2001). The gut addressin MAdCAM-1 (see below) is widely expressed in fetuses already at week 7. In fetuses, not only is it crucial for lymphocyte adhesion to vessels in gut, but it is also the most important determinant for lymphocyte binding to HEVs in peripheral lymph nodes. Although the adult peripheral node addressin, PNAd (see below), is expressed in humans at week 15, it only gradually starts to dominate in mediating lymphocyte trafficking to peripheral lymph nodes during early childhood. On the basis of these findings it is likely that efficient lymphocyte recirculation takes place already in utero.

TRAFFICKING OF LYMPHOCYTES TO SECONDARY LYMPHOID ORGANS

Peripheral lymph nodes, Peyer's patches, and spleen are the prime target organs for naïve lymphocytes. In lymph nodes, HEVs, based on the characteristic cuboidal morphology of endothelial cells, allow efficient recruitment of bloodborne lymphocytes (Kraal and Mebius, 1997; von Andrian and Mempel, 2003). In these postcapillary venules, the shear stress is relatively low, and the endothelial surfaces protruding into the lumen of the vessels are rich in specific molecules needed for efficient adhesion. Moreover, the junctions between endothelial cells in these vessels are not typical tight junctions; rather, a flap-valve–like mechanism is operative at this site, which also facilitates cellular transmigration. Consequently, when the lymphocytes flow into HEVs they undergo the different steps of the classical extravasation cascade very efficiently. It has been estimated that up to 50 percent of incoming lymphocytes make contacts with the HEV lining in Peyer's patches, and that even one cell in four can ultimately extravasate in these specialized organs (Bjerknes et al., 1986). On the basis of calculations in sheep, up to 15,000 lymphocytes can be expected to be transmigrating through HEVs in a single lymph node every second (Girard and Springer, 1995).

Antigens are concentrated to lymph nodes through the other vessel systems draining into these organs (Randolph et al., 2005). Free antigens or antigens taken up by dendritic cells enter lymph nodes from the periphery via afferent lymphatic vessels. These vessels empty into the subcapsular sinuses, from which cells and antigens can penetrate into the lymphoid areas while flowing through the fine lymphatic channels within the tissue. The dual vessel supply bringing in lymphocytes by blood and antigens by lymph maximizes the likelihood for lymphocyte–antigen contacts in lymph nodes.

When lymphocytes enter the lymphoid tissue they are further guided by chemotactic gradients into specific intra-

organ locations. Most notably, different chemokines secreted by many cell types help to segregate lymphocytes into discrete T- and B-cell areas within the lymph node (Sallusto and Baggiolini, 2008). Thus B cells preferentially accumulate into cortical B-cell follicles, whereas T cells are recruited into more diffuse subcortical T-cell areas. While percolating through the tissue stroma the lymphocytes constantly search for their cognate antigens presented by different types of professional antigen-presenting cells also concentrated in lymph nodes. If they fail to find a specific antigen in the lymph node, they enter the lymphatic sinuses and leave the organ via efferent lymphatic vessels. These cells are then carried back to the blood system, since the main lymphatic trunks (such as the ductus thoracicus) open into the major veins near the heart. Then these cells are free to move on in the circulation and to enter any other lymphoid tissue in the body during their continuous recirculation (Gowans and Knight, 1964). One cycle from the blood back to the blood takes about 1 day, so any single lymphocyte has time to percolate through hundreds of lymph nodes until it dies. This patrolling behavior of a lymphocyte is interrupted and retargeted only if it encounters its specific antigen within a lymphoid tissue (see below).

Lymphocyte recirculation through the spleen is profoundly different from that in the lymph nodes (Mebius and Kraal, 2005). Notably, HEVs are absent from the spleen and lymphocytes enter the white pulp via marginal-zone sinuses, where macrophage-like cells surrounding the endothelial cells may be important for recruitment. In the splenic parenchyma, lymphocytes again migrate toward specific guidance molecules. However, here T cells migrate into confined T-cell areas around the central arteriole, whereas B cells remain scattered in the corona. Splenic lymphocytes also seem to leave the organ via the splenic vein rather than via lymphatics. Although the spleen contains more lymphocytes than all the lymph nodes together, the migratory pathways in this organ specialized for concentrating blood-derived antigens remain rather poorly characterized.

TISSUE-SELECTIVE HOMING OF ACTIVATED LYMPHOCYTES

When a lymphocyte meets its cognate antigen in lymph nodes, its migratory properties change profoundly (Gowans and Knight, 1964; von Andrian and Mempel, 2003). The activated lymphocyte undergoes proliferation and differentiation in the lymph node. Thereafter, its progeny cells leave the node via the efferent lymphatics together with other naïve lymphocytes. However, upon re-arrival into the circulation, the antigen-activated effector cells no longer randomly recirculate to other lymph nodes. Instead, they have become imprinted so that they can preferentially traffic into the peripheral epithelial tissues through which the inciting antigen penetrated the body surfaces. This reprogramming is mainly instructed by local dendritic cells, which adapt external cues into signals giving information on the tissue localization (Sigmundsdottir and Butcher, 2008). The imprinting involves at least changes in the expression profile of adhesion molecules and receptors for chemokines as well as effector functions of lymphocytes. These effector cells can leave the blood via the normal flat-walled postcapillary venules in the periphery and navigate into areas of microbial or other inflammatory stimuli. Hence, the effector cells are efficiently targeted to those locations, where they can do the most good to defend the body.

A small population of effector cells ultimately turns into memory cells. Should the same antigenic exposure reoccur, the memory cells can evoke a rapid response against it. These cells can be divided into central memory and effector memory cells, which have different abilities to migrate into lymphoid and nonlymphoid tissues (such as epithelial surfaces). The central memory cells can home back to lymph nodes via HEVs, whereas the effector memory cells have mainly lost this ability and migrate to the peripheral tissues instead. Again, the phenotypic changes in the memory cell subsets reflect their recirculation potential.

Under physiological conditions, two main recirculation routes for activated lymphocytes can be distinguished (Fig. 7.1) (reviewed in Agace, 2008; Butcher and Picker, 1996; Salmi and Jalkanen, 1997; Springer, 1994; von Andrian and Mempel, 2003). Immunoblasts activated in peripheral lymph nodes preferentially migrate into the skin, whereas those responding to gut-derived antigens in Peyer's patches tend to return to the gut-associated lymphatic tissues. The gut-seeking immunoblasts enter mainly the intestine in the lamina propria, which is the body's largest nonorganized lymphoid tissue. These cells may also have the capacity to migrate into the respiratory and genitourinary tract, which have traditionally been included in the common gut-associated lymphoid system (Brandtzaeg and Johansen, 2005). Thus, under normal conditions, the gut and nonmucosal lymphoid organs share the total pool of naïve cells with all possible antigenic specificities, but upon activation, the subsequent migratory route sharpens the immune response into the affected tissue.

During pathological inflammation there are characteristic changes in leukocyte trafficking (Butcher and Picker, 1996; Ley et al., 2007; Salmi and Jalkanen, 1997; Springer, 1994). First, in acute inflammation, polymorphonuclear leukocytes invade the affected site very rapidly, and only later are waves of monocyte and lymphocyte influx seen. In a primary challenge it takes about 3 days before antigen-specific immunoblasts enter the peripheral lesion, but during a secondary response the influx of memory-derived cells occurs much faster. Second, the exposure of flat-walled venules to proinflammatory mediators at a site of acute inflammation makes them much more adhesive toward different classes of leukocytes. Bathing of the affected tissue in inflammatory cytokines, microbial products, and other pro-adhesive compounds results in the rapid synthesis of many endothelial adhesion molecules that are absent from non-inflamed vessels or expressed only at low levels. In chronic inflammation, even an HEV-like transformation of vessels in peripheral tissues is common and is quite often associated with notable lymphoid follicles in these nonlymphoid organs.

Different types of inflammations have typical features that also affect lymphocyte trafficking (Ley et al., 2007). In general, CD8-positive cytotoxic T cells and CD4-positive Th1, Th2,

Th17, and T reg cells, as well as different B-cell subsets, can infiltrate to sites of inflammation, but their relative proportions vary according to the underlying cause. The inflammatory stimulus and/or anatomical location results in a characteristic, although partially overlapping, pattern of expression and/or activation of adhesion molecules and chemotactic systems on both lymphocytes and endothelial cells, which translates into apparently tissue-selective migration patterns into inflamed tertiary sites. For example, inflamed synovial membrane in rheumatoid arthritis displays features of lymphocyte homing, which are clearly distinct from those in peripheral lymph nodes, skin, and gut. In fact, restricted expression of homing-associated molecules on Th1 versus Th2 CD4 lymphocytes may explain the preponderance of Th1 cells in certain inflammatory lesions (D'Ambrosio et al., 2000; Syrbe et al., 1999).

MOLECULAR MECHANISMS IN LEUKOCYTE EXTRAVASATION

THE MULTISTEP ADHESION CASCADE

Lymphocytes and other leukocytes leave the blood in a multistep process that can be visualized both in vitro and in vivo as tethering, rolling, adhesion, crawling, and transmigration (Fig. 7.2) (Butcher, 1991; Springer, 1994; Zarbock and Ley, 2009). Tethering and rolling phases are mainly mediated by selectins and their sialomucin ligands. Transient and weak interactions between lymphocytes and endothelial cells formed by these molecular pairs lead to triggering of an activation step involving chemokines and their receptors. Activation is required for optimal adhesive behavior of leukocyte integrins that are inactive when cells are freely flowing in the bloodstream. However, engagement of other types of molecules, such as glycosyl-phosphatidylinositol (GPI)-linked proteins, on the lymphocyte surface can also result in integrin activation. During activation and firm adhesion, leukocytes change their shape, crawl actively on endothelium, and then start to transmigrate. Molecular mechanisms functioning at the transmigration step have remained poorly known and there has been continuous debate during the past 40 years whether the leukocytes use a transcellular or paracellular route during the transmigration (Engelhardt and Wolburg, 2004). Currently it is thought that in principle the leukocytes are capable of using both transmigration routes, but the leukocyte subtype, the vascular bed, and the inflammatory status of the tissue determine the transmigration route (Sage and Carman, 2009). In both routes the path used by a transmigrating lymphocyte is rapidly closed by unknown repair mechanisms. Movement and final localization of a lymphocyte within the tissue is largely dependent on chemokine receptors and molecules binding to extracellular matrix proteins on its surface (Cyster, 1999, 2005).

The complexity of the multistep cascade functioning in leukocyte extravasation is comparable to blood clotting and complement-mediated killing, in which each step has to be executed correctly to achieve the goal. The extravasation cascade is made more complex by the fact that vascular beds in different organs and inflammatory conditions express some unique homing-associated molecules, and each leukocyte subset displays partially distinct homing molecules on its surface. Because the lymphocyte can enter a particular tissue only if it is equipped by a set of molecules matching the ligands presented on the vascular endothelium of this tissue, the composition of lymphocytic infiltration in each tissue is tightly controlled (Butcher, 1991; Salmi and Jalkanen, 1997; Springer, 1994).

The most relevant homing-related molecules (illustrated in Fig. 7.3) are presented below in more detail and in the order of their principal place in the adhesion cascade. It should be emphasized here, however, that the functions of these molecules overlap in vivo and may depend on the characteristics of the vascular bed, where the interactions take place. For example, integrins are able to mediate rolling under low shear, and in narrow capillaries they may act without a clear preceding, selectin-mediated rolling phase (Salmi and Jalkanen, 1997; Springer, 1994).

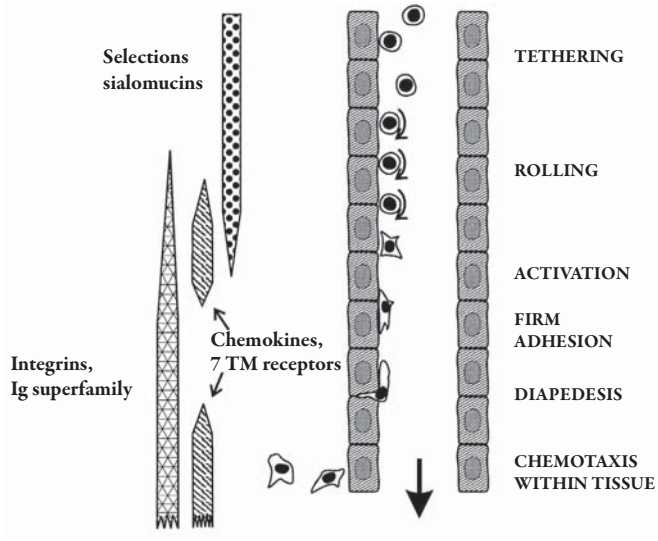

Figure 7.2 The multistep adhesion cascade of lymphocyte extravasation. The freely flowing, bloodborne lymphocyte starts the interaction with vascular endothelium by tethering and rolling; this proceeds to an activation step. Activation is followed by firm adhesion. Thereafter, the lymphocyte seeks an interendothelial junction, through which it penetrates into the tissue. The contribution of major molecular families at each step is illustrated.

TETHERING AND ROLLING

Selectins and Their Sialomucin Ligands

Selectins and their sialomucin ligands are the key players in tethering and rolling steps because the lectin-carbohydrate bonds between these receptor–ligand pairs allow formation of rapid but transient and weak interactions typical of rolling behavior. There are three members of the selectin family that have been named based on their main site of expression: L-selectin (CD62L) is present on different types of leukocytes, E-selectin (CD62E) is on endothelium, and P-selectin (CD62P) is on platelets and endothelium (Rosen, 2004; Vestweber and Blanks, 1999).

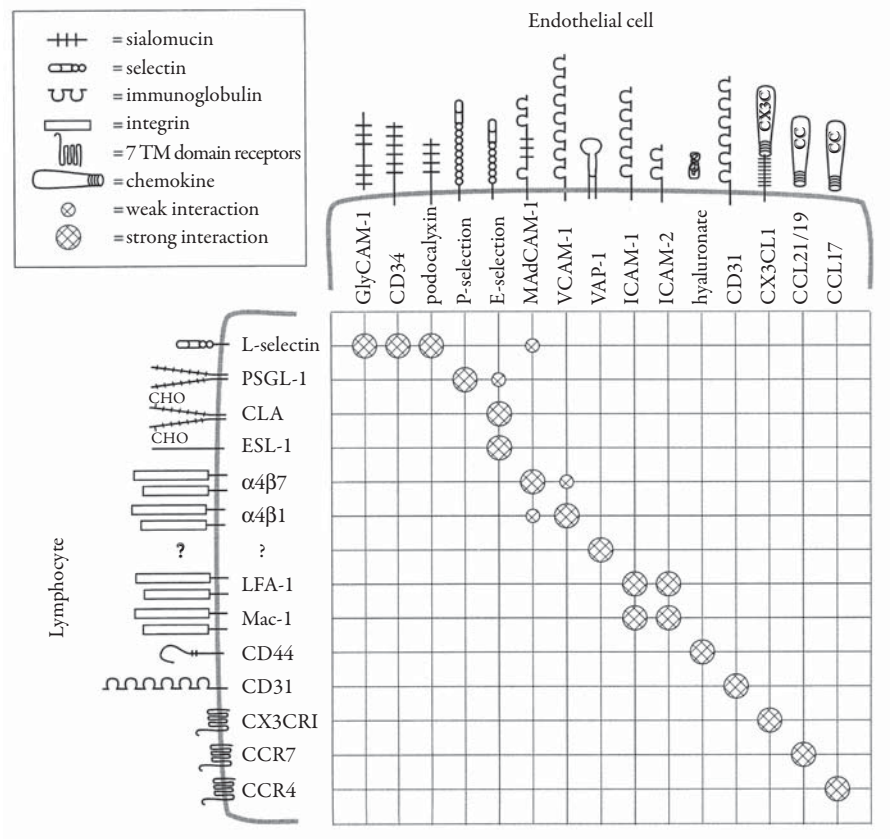

Figure 7.3 Homing-associated molecules involved in leukocyte traffic. The most relevant leukocyte surface molecules and their counter-receptors on vascular endothelium are depicted.

The common structural feature of these molecules is the N-terminal lectin domain, which is of fundamental importance in binding to sialyl Lewis X (sLeX) carbohydrate present on the sialomucin ligands of selectins (Fukuda et al., 1999; Rosen, 2004; Vestweber and Blanks, 1999). The ligand structures for L-selectin are presented by PNAd on HEVs in peripheral lymph nodes; thus, PNAd is fundamental to guiding L-selectin–positive lymphocytes into peripheral lymph nodes. PNAd consists of at least six different proteins, all decorated by a sulfated and fucosylated sLeX. They are glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, podocalyxin, nepmucin, endomucin, and MAdCAM-1. Interestingly, MAdCAM-1 has the proper glycosylation for L-selectin recognition only in organized lymphoid areas of the gut such as Peyer's patches but not in lamina propria vessels, allowing to a certain extent L-selectin–dependent entrance of cells to mucosal sites.

The importance of most homing-related molecules in vivo has been demonstrated by creating knockout mice (Table 7.1). Among the first were L-selectin–deficient mice, which exhibit severely impaired lymphocyte homing to peripheral lymph nodes. In contrast, GlyCAM-1 knockout mice only have enlarged lymph nodes, a result suggesting a regulatory role for the soluble GlyCAM-1 molecule in lymphocyte homing. CD34 knockout mice are seemingly healthy despite defective eosinophil migration. Also, the genes important for proper posttranslational modifications of the selectin ligands have been disrupted. For example, fucosyltransferase VII (FucTVII)–deficient mice are not able to add the critical fucose moiety to the sLeX structure. Hence, they do not glycosylate L-selectin ligands properly and exhibit severe impairment in lymphocyte homing and leukocyte extravasation to sites of inflammation (Lowe, 2003). As another example, lack of a highly HEV-specific sulfotransferase (GlcNAc6ST) leads to inefficient or absent sulfation of the sLeX motif, concomitant disappearance of L-selectin ligand activity, and decreased lymphocyte homing (Hemmerich et al., 2001).

Expression of both P- and E-selectin is induced at sites of inflammation. However, their expression kinetics differs remarkably. P-selectin is released within minutes from intracellular storage granules, Weibel-Palade bodies, and is translocated to the endothelial cell surface, whereas E-selectin requires new protein synthesis, and its maximal expression is seen 4 hours after the induction of inflammation. The primary ligand for P-selectin is correctly tyrosine-sulfated P-selectin glycoprotein ligand-1 (PSGL), which is present on most leukocytes (McEver and Zhu, 2010). Depending on its glycosylation profile it can also bind to E-selectin, which has high affinity particularly toward cutaneous lymphocyte antigen (CLA), a specific glycoform of PSGL-1. Interaction between CLA and E-selectin appears to be important in directing lymphocytes to skin inflammations. In addition, E-selectin

Table 7.1 PHENOTYPE OF GENE-TARGETED MICE MADE DEFICIENT FOR INDIVIDUAL HOMING-ASSOCIATED MOLECULE

MOLECULE	MAIN ABNORMALITIES IN LEUKOCYTE TRAFFICKING
L-selectin	Impaired lymphocyte homing to peripheral lymph nodes
E-selectin	Increased velocity of rolling leukocytes
P-selectin	Decreased leukocyte rolling at early stages of inflammation
GlyCAM-1	Enlarged lymph nodes
CD34	Impaired eosinophil accumulation in allergen-induced lung inflammation
PSGL-1	Decreased leukocyte rolling at early stages of inflammation
Fuc-TVII	Reduced leukocyte migration to sites of inflammation
	Reduced lymphocyte homing to peripheral lymph nodes
C2β GlcNAcT	Reduced leukocyte emigration to sites of inflammation
GlcNAc6ST	Decreased lymphocyte homing
ICAM-1	Impaired neutrophil migration to sites of inflammation
ICAM-2	Stimulus-specific decrease in granulocyte transmigration
VCAM-1	Embryonic lethal
ESAM	Reduced neutrophil extravasation to sites of inflammation
JAM-A	Increased dendritic cell homing to lymph nodes and reduced transendothelial cell migration of JAM-A–negative neutrophils
JAM-C	Reduced leukocyte adhesion to inflamed endothelium and transmigration
CD31	Deficient migration through basement membrane
CD11a	Reduced lymphocyte trafficking to lymphoid organs Decreased leukocyte trafficking to sites of inflammation
CD11b	Decreased leukocyte trafficking to sites of inflammation
CD11c	Decreased leukocyte trafficking to sites of inflammation
β2-integrin	Decreased leukocyte migration to sites of inflammation
α4-integrin	Embryonic lethal, chimeric mice have reduced lymphocyte homing into Peyer's patches
β7-integrin	Impaired lymphocyte migration to Peyer's patches
CD43	Increased lymphocyte homing to secondary lymphoid organs
CD44	Decreased lymphocyte migration to peripheral lymph nodes and thymus
VAP-1	Diminished leukocyte infiltration to sites of inflammation
CD73	Increased leukocyte traffic to sites of inflammation
CD38	Defective chemotaxis of leukocytes to severe inflammations
CCR4	Impaired T-cell trafficking to mesenteric lymph nodes and lung
CCR7	Defective lymphocyte entry to lymph nodes and formation of T-cell areas
CCR9	Reduced number of intraepithelial T cells and IgA-positive plasma cells in lamina propria
CCL19/CCL21	Impaired homing of lymphocytes (especially T cells) to lymph nodes
CXCR5/CXCL13	Defect in formation of follicles in lymph nodes
CX3CL1	No obvious defects in leukocyte trafficking

binds to a non-mucin-like E-selectin ligand-1 (ESL-1) and CD44 on leukocytes (Hidalgo et al., 2007; Vestweber and Blanks, 1999).

E-selectin knockout mice are apparently healthy, but their leukocytes roll faster than in normal mice, indicating that E-selectin is an important molecular brake at early steps of leukocyte–endothelial cell interactions. P-selectin–defective mice are also viable and fertile. They show neutrophilia due to a longer half-life of neutrophils. After induction of inflammation, leukocyte rolling is impaired for less than 2 hours in these mice. This finding suggests that P-selectin is indispensable only at early phases of inflammation (Frenette and Wagner, 1997). PSGL-1 knockout mice largely resemble P-selectin–deficient mice, thus confirming the importance of PSGL-1 at the rolling step (Yang et al., 1999). Mice deficient for both E- and P-selectin have also been generated. These mice exhibit much more profound defects than those of single knockouts, indicating that E- and P-selectin have overlapping

functions and that they compensate each other (Frenette and Wagner, 1997). Furthermore, mice whose core 2β 1, 6-*N*-*acetylglucosaminyl* transferase (C2-β-ClcNAcT) gene has been disrupted have a defect in the early step of branching of LeX oligosaccharide and therefore lose proper glycosylation of their E- and P-selectin ligands. These mice show normal lymphocyte homing to lymph nodes, but their leukocytes cannot efficiently enter the sites of inflammation (Etzioni et al., 1999; Vestweber and Blanks, 1999). A human immunodeficiency disease, leukocyte adhesion defect 2 (LAD2), resembles characteristics of these knockout animals: LAD2 patients do not have functional selectin ligands because of a glycosylation defect. Leukocytes of these patients have decreased rolling capacity, although in static conditions leukocytes adhere efficiently to endothelial cells. Patients with LAD2 suffer from recurrent bacterial infections and also have other developmental abnormalities (Etzioni, 2007; Notarangelo and Badolato, 2009). This disease is presented in detail in Chapter 38.

CD43, CD44, and Vascular Adhesion Protein-1

Other (nonselectin) molecules have also been shown to directly mediate rolling or tethering or to indirectly regulate this step. An example of a regulatory molecule is CD43, a long and negatively charged glycoprotein on lymphocytes that inhibits engagement of L-selectin, thus blocking the homing of lymphocytes. Its role is clearly demonstrated in CD43-deficient animals, which show enhanced lymphocyte homing to secondary lymphoid organs (Stockton et al., 1998). Although neutrophil CD43 generally serves as an antiadhesive molecule to attenuate neutrophil-endothelial interactions, it may have a proadhesive role as an E-selectin ligand (Matsumoto et al., 2008).

CD44 is a multifunctional member of the proteoglycan family that can mediate rolling of lymphocytes on endothelial hyaluronan and E-selectin (Hidalgo et al., 2007; Siegelman et al., 1999). It can be physically associated with VLA-4 on activated T cells to mediate efficient rolling and subsequent firm adhesion (Steeber et al., 2005). In addition, triggering of CD44 activates lymphocyte function–associated antigen-1 (LFA-1), which may strengthen lymphocyte adhesion to endothelium. In vivo experiments using function-blocking antibodies suggest that CD44 is important in lymphocyte trafficking to sites of inflammation. Moreover, lymphocytes of CD44-deficient mice show impaired lymphocyte homing to peripheral lymph nodes and thymus and diminished granulocyte influx to arthritic joints (Protin et al., 1999; Sarraj et al., 2006).

Vascular adhesion protein-1 (VAP-1) is a dual-function homing-related molecule, because in addition to its adhesive function at an early phase of the multistep cascade it possesses intrinsic enzymatic activity. The end products of the reaction catalyzed by this amine oxidase are aldehyde, ammonium, and hydrogen peroxide. All of them are potent compounds that may regulate the entire scene of inflammation by, for instance, aberrantly glycosylating endothelial proteins (aldehydes) or by upregulating other adhesion proteins and metalloproteinases and inducing apoptosis (hydrogen peroxide). Regulation of VAP-1 function appears to involve its sequestration into intracellular granules, from which it is rapidly translocated to the endothelial surface upon induction of inflammation. Mice lacking VAP-1 show mild impairment in lymphocyte homing to mucosal sites and reduced response to oral immunization. Moreover, leukocyte migration to sites of inflammation is diminished (Salmi and Jalkanen, 2005).

ACTIVATION

Chemokines and Their Receptors

Certain endothelial chemokines and their interaction with the seven pass transmembrane receptors on leukocytes are considered to be of fundamental importance at the activation step of the adhesion cascade. Chemokines are small molecules divided into the following families on the basis of their structural cysteine motifs: C, CC, CXC, and CX3C, where *C* is cysteine and *X* is any amino acid residue.

Chemokines are typically heparin-binding soluble molecules, and many of them are not even produced by endothelial cells. Nevertheless these molecules can exert their function at the luminal surface of endothelial cells, where blood constantly flushes away any soluble gradients. It seems that these molecules are carried via a specialized reticular conduit through the lymph node to the abluminal side of the vessels. They can then be transcytosed to the luminal side of the endothelial cell, where they become immobilized by an avid binding to surface-expressed glycosaminoglycans. In fact, chemokines injected subcutaneously can find their way to the luminal surface of HEVs in the draining lymph nodes, and hence selectively enhance lymphocyte recruitment to the draining node. After chemokine binding, activation signals are transduced from the serpentine receptors via guanine nucleotide-binding (G) proteins via complex signaling routes that eventually result in affinity/avidity changes of leukocyte integrins (Bromley et al., 2008; Colditz et al., 2007; Sallusto and Baggiolini, 2008).

Different leukocyte subsets possess alternative sets of chemokine receptors, which determine whether they are allowed to exit from the blood—for example, at HEVs in peripheral lymph nodes or in vasculature at sites of inflammation. For instance, T cells bearing CCR7 receptor become recruited to peripheral lymph nodes and mucosal sites where its ligands, CCL21 and /or CCL19, are expressed on HEVs. B cells, in contrast, use CXCR4 and 5 to bind CXCL12 and CXCL13, respectively. CX3CL1 (Fractalkine) is structurally very different from other endothelial chemokines because in addition to a soluble form it has a transmembrane form with a long stalk of sialomucin-like structure. CX3CL1 has a strong affinity toward CX3CR1-expressing leukocytes. Tissue selectivity among chemokines has been demonstrated to exist at least in the skin, where endothelial CCL17 and CCL22 attract CCR4-bearing lymphocytes, and in the small intestine, where CCR9-positive lymphocytes seek for CCL25 (Salmi and Jalkanen, 2005).

CCR7 knockout mice demonstrate the importance of this chemokine in lymphocyte trafficking, as these mice have defective T-cell entry to lymph nodes (Cyster, 2005). CCR9-deficient mice have a reduced number of mucosal lymphocytes, and mice lacking CXCR4 or CXCR5 have defects

in the formation of lymphoid follicles (Salmi and Jalkanen, 2005). In contrast, no obvious homing defects have been found in CX3CL1 knockout mice. The only abnormality discovered in CX3CL1$^{-/-}$ mice is the diminished number of cells belonging to the monocyte/macrophage lineage in the blood. In CX3CR1 deficiency the accumulation of dendritic cells is diminished in atherosclerotic lesions. CCR4-deficient T cells show impaired homing to mesenteric lymph nodes and inflamed lungs.

CD73 and CD38/CD157

Engagement of several other leukocyte surface molecules can lead to activation of LFA-1, CD 11 a/CD 18. However, only a few of them have been directly shown to result in increased leukocyte binding to endothelium. One of the molecules having this property is CD73, a GPI-linked molecule that is expressed on endothelium and on a small subset of lymphocytes and has ectonucleotidase activity catalyzing conversion of extracellular AMP to adenosine (Colgan et al., 2006; Jalkanen and Salmi, 2008). Adenosine regulates E-selectin expression, integrin function, and endothelial permeability (Cronstein, 1997; Cronstein and Weissmann, 1993). Interestingly, lack or diminished expression of CD73 is linked to a variety of immunodeficiency diseases, such as Wiskott-Aldrich syndrome, severe combined immunodeficiency, common variable immunodeficiency, primary hypogammaglobulinemia, selective IgA deficiency, and Omenn syndrome. Mice lacking CD73 are prone to more severe vascular leakage in hypoxic and ischemic conditions than their wild-type counterparts, confirming the role of CD73 in maintaining endothelial cell barrier function (Jalkanen and Salmi, 2008).

CD38 and CD157 are close relatives and have both ADP-ribosyl cyclase and NAD-hydrolase activities. They are expressed in most human leukocytes and can modulate leukocyte trafficking by regulating the sensitivity of cells to chemokines, calcium influx, and thus integrin function through inside-out signaling. CD38-deficient mice have defective chemotaxis. Patients suffering from paroxysmal nocturnal hemoglobinuria lack CD157 and their neutrophils have constant defects in adhesion and migration (Deaglio and Malavasi, 2006).

FIRM ADHESION

Integrins and Their Immunoglobulin Superfamily Ligands

Certain integrins participate effectively in the arrest of leukocytes on the endothelium through use of members belonging to the immunoglobulin superfamily as their vascular ligands (Denucci et al., 2009). They can also function at the earlier steps of the cascade, even during rolling. Typical for integrins is that they are heterodimers consisting of α and β chains. Important integrins for leukocyte extravasation are α4β7, LFA-1, and α4β1. α4β7 is a principal homing receptor for MAdCAM-1 and directs lymphocytes to mucosa-associated lymphatic tissues (Butcher, 1999). Its role as a mucosal homing receptor has been clearly demonstrated by β7 knockout animals. They have rudimentary Peyer's patches and their lymphocyte homing to mucosal sites is severely impaired. In contrast, lymphocyte homing to peripheral lymph nodes in these mice is intact (Butcher, 1999).

α4β1, in contrast, exerts its role mainly at inflammatory sites, where it binds to its ligand, vascular cell adhesion molecule-1 (VCAM-1, CD106). Both VCAM-1 and α4 knockout animals are embryonically lethal, indicating that besides homing they have more fundamental roles in embryonic development. Mice that are chimeric regarding the deletion of α4 show diminished lymphocyte trafficking to Peyer's patches but not to other secondary lymphatic organs, a finding compatible with the phenotype of β7 knockouts (Butcher, 1999).

LFA-1 (CD11a/CD 18) is a member of the group of four leukocyte integrins sharing the same β chain, β2 (CD 18), while having unique α chains (CD 11a, b, c, and d). LFA-1 is present on practically all leukocytes and mediates their binding to intercellular adhesion molecules 1 and 2 (ICAM-1 [CD54] and ICAM-2 [CD 102]) in its active form (Alon and Dustin, 2007). Mac-1 (CD11b/CD18) also participates in leukocyte migration, but its role is overshadowed by LFA-1. The role of leukocyte integrins in leukocyte trafficking is evident through the dramatic defects in leukocyte extravasation in patients suffering from LADI or LADIII. LADI patients lack the normal β2 chain and consequently surface expression of all leukocyte α chains is prevented, or they express LFA-1 in a nonfunctional form. In LADIII all hematopoietic integrin activation processes are defective and besides severe infections the patients suffer from increased bleeding tendency (Etzioni, 2010). These deficiencies are presented in more detail in Chapter 38 in this book.

Mice deficient in CD18 do not show as dramatic defects as LAD1 patients. Their neutrophils do not migrate properly to sites of inflammation. CD1la$^{-/-}$ mice have impaired lymphocyte homing to both peripheral lymph nodes and Peyer's patches, a finding in keeping with the role of LFA-1 as a non–organ-specific homing molecule. In CD11b- and CD11c-negative mice, reduced leukocyte trafficking to sites of inflammation is observed. Mice lacking ICAM-1 have a relatively mild phenotype compared to findings obtained from studies using function-blocking antibodies. These mice have decreased neutrophil migration to tissues in some inflammatory models. Lack of ICAM-2 normally constitutively present on endothelium does not cause any obvious defects in lymphocyte recirculation.

Crawling and Transmigration

Transmigration is the least-analyzed phase in the leukocyte extravasation process, although recent progress has been made (Carman and Springer, 2008). Before actual transmigration the leukocytes crawl on endothelium, actively seeking a proper site to enter the tissue (Kelly et al., 2007). Studies using function-blocking antibodies have shown that CD31 is intimately involved in transmigration. CD31 belongs to the immunoglobulin superfamily and is expressed by several leukocyte subsets and continuous endothelium of all

vessel types. On the vascular endothelium its expression is concentrated on intercellular junctions. Unexpectedly, CD31-deficient mice show a normal overall transmigration capacity. However, the polymorphonuclear leukocytes are arrested between the endothelium and basement membrane, demonstrating that CD31 is needed in migration through the basement membrane (Woodfin et al., 2007). Other molecules such as junctional adhesion molecules (JAMs) and their close relative endothelial cell-selective adhesion molecule (ESAM), as well as CD99, LFA-1, and the ICAMs, have been implicated in transmigration via the paracellular route (Ley et al., 2007; Weber et al., 2007). Also matrix metalloproteinase-2 (MMP-2), which is induced in T cells upon binding to endothelium, and MMP-9 play a role in this invasive process. Overall, the current evidence suggests that also the transmigration requires stepwise and carefully regulated interactions of the molecules involved.

There are still limited amount of data about the molecular mechanisms operating in the transcellular transmigration route. ICAM-1 and VCAM-1 are both participating in formation of the transmigratory cap around the extravasating leukocyte (Carman and Springer, 2008). Also plasmalemmal vesicle-associated antigen (PV-1) functions in the transendothelial route (Keuschnigg et al., 2009).

LYMPHOCYTE TRAFFIC VIA THE LYMPHATICS

The molecular mechanisms mediating leukocyte migration via the afferent lymphatics into the draining lymph nodes and lymphocyte exit via the efferent lymphatics are less well known than those controlling leukocyte extravasation from the blood into the tissues. Recently, however, several molecular interactions have been reported to contribute to migration of dendritic cells and lymphocytes through afferent lymphatics. Those include CCL21 chemokine presented on lymphatic endothelium, sphingosine 1 phosphate receptor 1 ($S1P_1$) expressed in many different cell types, JAM-A, ICAM-1, VCAM-1, Clever-1/Stabilin-1 and MR. CCL21 attracts CCR7-positive dendritic cells and lymphocytes into the afferent lymphatics and further to the draining lymph nodes. $S1P_1$, on the other hand, plays a dual role: it participates in lymphocyte entrance into the draining lymph nodes via afferent lymphatics and in their exit from the nodes via efferent lymphatics. It also regulates emigration of thymocytes from the thymus (Debes et al., 2005; Rosen and Goetzl, 2005). The SIP_1-deficient mice are embryonically lethal due to defects in vascular development. However, conditional knockout mice have confirmed the role of SIP_1 in lymphocyte egress from the lymph nodes into the efferent lymphatics. Recent studies suggest that SIP_1 controls lymphocyte trafficking by regulating endothelial permeability and/or suppressing lymphocyte chemotaxis in SIP gradients (Cyster, 2005; Rosen and Goetzl, 2005). Clever-1/Stabilin-1, a scavenger present both on afferent and efferent lymphatics, contributes to lymphocyte traffic via the lymphatics. Junctional adhesion molecule-A (JAM-A) is also involved in cell trafficking within lymphatics as dendritic cells from JAM-A–deficient mice show an increased capacity to transmigrate across lymphatic endothelial cells and altered trafficking to the lymph nodes. Interestingly, the altered migration was found only when the dendritic cells lacked JAM-A but not in the case of endothelium-restricted deficiency of JAM-A. Moreover, ICAM-1 and VCAM-1 are induced on lymphatics upon inflammation and mediate dendritic cell trafficking into the draining lymph nodes (Johnson and Jackson, 2008). CCR7, $S1P_1$, JAM-A, Clever-1/Stabilin-1, VCAM-1, and ICAM-1 are all expressed on a wide variety of other cell types in addition to lymphatic endothelium, and they all are also involved in leukocyte extravasation through the vascular endothelium. In contrast, macrophage mannose receptor (MR) is one of the few adhesion molecules that is expressed on lymphatic but not on blood vessel endothelium. Mice lacking MR have impaired lymphocyte trafficking via afferent lymphatic vessels into the draining lymph nodes, demonstrating the role of MR in lymphocyte migration via lymphatics (Marttila-Ichihara et al., 2008).

INTRAORGAN LOCALIZATION OF LYMPHOCYTES

Subsequent to entry into the lymphoid organs, lymphocytes start their ameboid voyage within the organs. For the migration, lymphocytes need balanced function of adhesion molecules capable of binding to extracellular matrix molecules (ECMs), such as fibronectin, laminins, and collagens, and mechanisms to detach from the anchorage to ECMs (Friedl and Weigelin, 2008). β1-integrins are essential for binding to several ECMs, and lymphocytes can use CD44 to bind to fibronectin and collagens. Detaching mechanisms are poorly understood. Controlled and directional migration also needs proper chemokine receptors on the lymphocyte surface that respond to chemokines secreted by different cell types within the tissues. These interactions are important both in early phases of lymphocyte maturation, for example in the thymus, and later during physiological lymphocyte recirculation and leukocyte movement at sites of inflammation (Bonecchi et al., 2009; Bromley et al., 2008). During normal recirculation, CCL21 and CCL19 guide T cells to interfollicular areas within the nodes, whereas CXCL13 produced by follicular dendritic cells attracts B cells expressing CXCR5 into the follicles (Fig. 7.4).

In addition to cell-surface molecules mediating adhesion–de-adhesion steps in the intraorgan movement of lymphocytes, the pathways that transduce these surface signals into the locomotory machinery are important for proper localization (Barreiro et al., 2007). Hence, specific receptors are clustered at the leading edge and trailing edge of the crawling lymphocyte. These molecules signal to the actomyosin and microtubuli networks within the cell, and translate the surface signals into polarized cell crawling. Just one example of the importance of these steps for proper immune defense is Wiskott-Aldrich syndrome, in which a defect in one of these effector signaling proteins, WASP, leads to a severe immunodeficiency (Bosticardo et al., 2009).

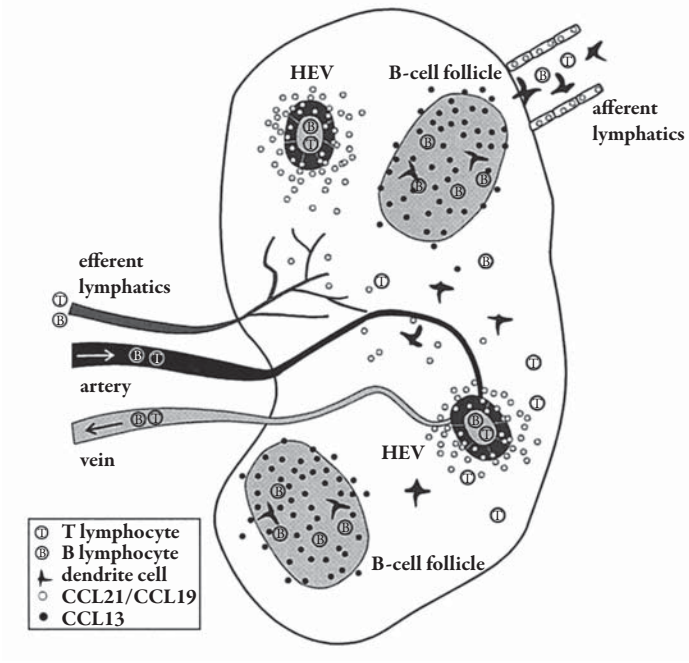

Figure 7.4 Chemokines contribute to both entrance and intraorgan localization of lymphocytes. CCL19 and CCL21 are involved in T-lymphocyte entrance via high endothelial venules (HEVs) into the node. They also guide T cells to the interfollicular areas in the lymph node. CXCL13, by contrast, directs B lymphocytes into the follicles.

Dendritic cells entering the lymph nodes via afferent lymph and bringing antigens are key players in the immune response as antigen-presenting cells. CCL21 expressed in lymphatic endothelium assists dendritic cell entrance into the T-cell areas, allowing these two cell types to interact with each other. B-cell collaboration with T cells is ensured by upregulation of CXCR5 on a subset of T cells and CCR7 on B cells. This makes it possible for B and T cells to move to the boundary of the B- and T-cell zones and interact with each other. A spontaneously mutant mouse strain, *plt*, which has decreased expression of both CCL21 and CCL19, demonstrates the importance of these chemokines in intraorgan localization (and entry) of lymphocytes, as these mice have defective organization of T-cell areas within lymph nodes. The phenotype of these mice is thus comparable to that of mice lacking the receptor for these chemokines (CCR7). In contrast, in CXCR5-negative mice development of B-cell follicles is defective—again, in keeping with the function of this chemokine in B-cell localization (Okada and Cyster, 2006).

Intraorgan localization also shows remarkable tissue specificity, best exemplified in the gut. Practically all lymphocytes in the small intestine have CCR9, whereas in the colon these cells are less frequent. Importantly, lymphocytes in most other tissues (excluding thymus) are CCR9 negative. CCR9 ligand, CCL25, is produced by gut epithelial cells and guides CCR9-positive lymphocytes, especially to the epithelium of the small intestine (Agace, 2008).

CONCLUSIONS

Optimal lymphocyte entry to primary lymphoid organs during development and to secondary lymphoid organs during recirculation and efficient leukocyte trafficking to sites of inflammation are the key elements in adequate functioning of the immune system. The molecular mechanisms mediating lymphocyte contacts with the vascular wall and governing the intraorgan localization of lymphocytes are relatively well known. Knowledge on the mechanisms functioning during diapedesis through the vascular wall, leukocyte entry to afferent lymphatics at different vascular beds, and lymphocyte exit via efferent lymphatics within the lymphoid organs is rapidly increasing.

The importance of leukocyte trafficking to the pathophysiology of human disease is best reflected by the rare genetic disorders of lymphocyte migration, and by the enormous potential of the recently introduced therapeutics modulating adhesion molecules in inflammatory diseases. Thus far, the number of patients diagnosed as having a defect in any of the molecules involved in leukocyte migration has been very small. One reason for this small number may be that many of the defects are not compatible with life. The tools for diagnosing patients who suffer from defective leukocyte trafficking are continuously being developed and may help to reveal many new disease entities. The adhesion and activation molecules have been tempting targets for anti-inflammatory drugs ever since they were discovered. During the past few years this research has translated into clinical success (Luster et al., 2005; Mackay, 2008). A humanized antibody against α4 (natalizumab) has proven to be highly effective, especially in the treatment of multiple sclerosis and Crohn's disease, respectively. Although a few serious side effects have been reported, it is actively and successfully used in clinics. Moreover, dozens of drugs targeting other adhesion molecules and chemokines are in late clinical trials.

Over the past few years, we have gained increasing insight into the salient molecular and physiological mechanisms guiding lymphocyte recirculation from the blood into the tissues and back to the circulation. In terms of our immune defense, this continuous patrolling process is important for guaranteeing maximal efficacy of a lymphocyte meeting its cognate antigen. Failure of this process inevitably leads to some form of immunodeficiency. Thus, a detailed understanding of the multifaceted extravasation cascade will help us to develop new strategies for guiding and redirecting lymphocyte trafficking into locations where the system has gone awry for one reason or another.

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8.

INNATE IMMUNITY

Jordan S. Orange, Michael M. Frank, and Stuart E. Turvey

The innate immune system serves as the initial immunological defense against foreign and dangerous material. Innate immunity is required once a challenge to the host organism has managed to penetrate certain physical defenses that the host presents as a barrier between itself and the environment. Examples of these physical defenses in humans include the skin and mucus. While the host physical barriers to danger are essential for survival, this chapter is focused upon the first line of immunological defense—the initial immune elements used in encountering challenges that have succeeded in overcoming the physical divisions between host and environment. As entire volumes are devoted to the topic of innate immunity, the summary provided here is intended to (1) define the general paradigms through which the innate immune system functions; (2) identify key components of the innate immune system and provide some insight into how they function in host defense; and (3) introduce how some of the innate immune elements critically link to the adaptive immune system to coordinate an integrated immunological defense.

PARADIGMS IN INNATE IMMUNITY

Unlike the adaptive immune system (see Chapters 4 and 5), the innate immune system is "hardwired" to recognize and respond to danger (Janeway and Medzhitov, 2002). In other words, innate immune elements do not require genetic recombination events or a developmental phase to mediate function. All of the function is encoded within the germline DNA and is poised to exert immediate or near-immediate effects. This includes not only recognizing the dangerous challenge, but also responding to it and acting to contain or eliminate the insult. Based on this immune recognition strategy, there are three important paradigms of the innate immune system that in some way apply to the majority of its components: recognition, amplification, and response.

In essence, each of these paradigms represents a phase of an innate immune response. During the recognition phase, danger is specifically identified within a complex environment containing endogenous and nondangerous materials. An example would be recognizing a bacterium that penetrates the skin on the surface of a sewing needle. In the amplification phase, the recognition of danger leads to the generation of a discernable signal. This can be intracellular, and occur within the same cell that had recognized the dangerous element, or extracellular. Extracellular amplification can occur at the level of direct intercellular contacts, or through soluble mediators that act at long distances. The amplification phase serves the purpose of expanding a one organism versus one defense interaction into an immune response potentially involving multiple defenses. The response phase of innate immunity depends on the amplified signal leading to a function directed at the offending stimuli. In the case of the bacterium, this could be its ingestion and destruction inside a phagocyte, or its direct dissolution through a soluble antibacterial molecule. Before defining the individual components of the innate immune system, the fluid concept of these three paradigms will be further discussed with specific reference to the types of immune elements responsible for each phase. While not exhaustive, the objective is to introduce the concept of the innate immune response so that the breadth and specificity of innate immunity can be appreciated (Beutler, 2009).

RECOGNITION PHASE OF INNATE IMMUNITY

A central function of the innate immune system is the rapid recognition of potentially harmful materials. These can

originate from the environment, as in the case of a pathogenic bacterium, or from within the host itself, such as a cancerous cell; in addition, the innate immune system has the capacity to sense tissue damage and some of the common biochemical consequences of infection. While the recognition of the element may immediately lead to its eradication, in some cases it represents only the very beginning of a multifaceted response aimed at containing and ultimately eliminating the threat. Two major strategies are used by the innate immune system in recognition: pattern recognition and danger sensing.

PATTERN RECOGNITION

Pattern recognition represents the ability of the innate immune system to recognize molecular motifs inherent to something challenging the host and to differentiate signals from motifs normally present throughout the host organism. As such, this is an essential feature of recognition within the innate immune system. It was originally defined by Janeway through his concept of *pathogen-associated molecular patterns* (PAMPs) (Janeway, 1989). PAMPs are characteristic hallmarks of pathogenic organisms that the immune system can exploit to distinguish them immediately from self-tissues. By definition, PAMPs are not a product of host DNA. Some examples include lipopolysaccharide (LPS) and double-stranded RNA. PAMPs technically fall into the broader category of *microbial-associated molecular patterns* (MAMPs). The distinction between a MAMP and a PAMP is useful as not all relevant patterns capable of being recognized are associated with pathogenic microorganisms. MAMPs can still be recognized by the innate immune system and present the ability to facilitate immunological responses and cellular functions. An alternative but equally relevant paradigm is the recognition of *danger-associated molecular patterns* (DAMPs) (Bianchi, 2007). DAMPs are not inherent to a pathogen and are products of host DNA. Because of their ability to elicit an immune response, however, they are generally contained within protected environments and are not routinely exposed to the immune system. DAMPs, however, may be induced directly, or indirectly by a pathogen or other signals of stress such as malignancy. An example of a DAMP would be heat shock protein-70, which is encoded in the human genome and can be induced under a variety of stressful circumstances. Another important example is that of DAMPs derived from mitochondria during celluar injury, which can promote inflammation and under severe circumstances link to systemic inflammatory response syndrome (Zhang, 2010).

A critical element for a molecular pattern to act as a stimulus for innate immunity is the presence of a specific means for recognizing the pattern. In this light, there are a number of host-encoded receptor systems that are used for the recognition of PAMPs, MAMPs, and DAMPs. Collectively, these define a family of innate immune receptors referred to as *pattern-recognition receptors* (PRRs). The best appreciated of the PRRs at this point are the Toll-like receptors (TLRs).

In humans there are 10 TLRs, which have been described to recognize over 45 foreign molecules and 17 endogenous molecules as ligands (well-characterized examples in Table 8.1) (Akira and Takeda, 2004; Beutler, 2009; O'Neill, 2008; Takeda et al., 2003). All TLRs are transmembrane proteins. Some of the TLRs are found on the cell surface, including TLRs 1, 2, 4, 5, 6, and 10. TLRs 3, 7, 8, and 9 are found intracellularly and are localized within the endosomal compartment. As a result, different TLRs are specialized in recognition of patterns characteristic of extracellular or intracellular environments. Exemplary microbial ligands of the TLRs include lipoaribinomannan (TLR1/2), fungal zymosan (TLR2±6), double-stranded RNA (TLR3), bacterial lipopolysaccharide, and RSV fusion protein (TLR4), bacterial flagellin (TLR5), mycoplasmal diacyl lipopeptides (TLR6/2), viral single-stranded RNA (TLR7 and 8), and unmethylated CpG islands within bacterial and viral DNA (TLR9). An exemplary host-derived ligand is HSP-70, which is capable of ligating TLR 2/6 and 4. TLRs can also recognize synthetic molecules, such as imidazoquinolines (TLR7 and 8), and this represents a growing field of actual and experimental therapeutics aimed at inducing innate immunity. The signaling that results after TLR ligation is complex and has the objective of inducing transcriptional activation. These signal events therefore represent an amplification and are discussed separately as an amplification paradigm.

Table 8.1 **EXAMPLES OF TLR LIGANDS**

TLR	LIGAND	SOURCE
TLR1/2	Lipoarabinomannan	Mycobacteria
TLR2±6	Zymosan *HSP70*	Fungi Host
TLR3	DS RNA	Viruses
TLR4	Lipopolysaccharide RSV fusion protein *HSP70*	Gram-neg. bacteria RSV Host
TLR5	Flagellin	Flagellated bacteria
TLR6/2	Diacyl lipopeptides	Mycoplasma
TLR7 and 8	SS RNA Imidazoquinolones	Viruses Synthetic
TLR9	Unmethylated CpG motifs	Bacteria and DNA viruses
TLR10	Unknown	

In addition to TLRs, there are a number of other important PRRs, which are increasingly appreciated for their role in host defense. These include the nucleotide-binding oligomerization domain (NOD) family (Benko et al., 2008; Chen et al., 2009; Ting et al., 2008). The best known of these is NOD2, which is mutated in a subset of individuals with Crohn's disease as well as in Blau syndrome (Haskins et al., 1988; Hugot et al., 2001; Masters et al., 2009). NOD2 binds to the muramyl-dipeptide of bacterial peptidoglycan and thus can initiate response amplification after recognizing intracellular bacterial components. Formyl peptide receptors bind formyl peptides such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), which is a recognizable byproduct of bacterial metabolism. Thus, recognition of fMLP can serve as an important chemoattractant

for innate immune cells, such as neutrophills, that express these receptors. The natural cytotoxicity receptors, NKp44 and NKp46, also function as PRRs and are appreciated for their inherent ability to bind viral hemagglutinins. They are expressed on natural killer (NK) cells and in animal models have been shown to be needed for defense against influenza.

In addition to the above-mentioned cell-surface and intracellular PRRs, there are also a number of soluble proteins that have preference for motifs possessed by microorganisms. In a sense these are PRRs as they recognize dangerous material through their inherent specificity and have the ability to access immune function. In some cases these molecules mediate function directly and are introduced as a group. A first example is lectins or collectins, a family of proteins that bind carbohydrates. Some of these have specific utility in binding microbial carbohydrates; a key example is mannan-binding lectin (MBL), which binds to carbohydrate structures containing mannose present on microorganisms. MBL utilizes associated MASP (MBL-associated serine protease) proteins to activate complement and amplify an immune response as well as inducing an antibacterial response. MBL is held by some authors to be important in defense against infection, as individuals deficient in their ability to express the molecule have been found to have infectious susceptibility (Thiel et al., 2006). There are, however, conflicting observations regarding the significance of MBL deficiency in humans. Finally, there are a number of antimicrobial peptides that maintain a selective ability to bind to microorganisms (Hancock and Sahl, 2006). These include the defensins, cathelicidins, and histatins. In general they are 15–45aa positively charged peptides that will preferentially associate with the dense phospholipids characteristic of microbial membranes and cell walls. These peptides can then insert into the microbe and have direct antiproliferative effects. They can in some cases lead to microbial destruction, which will expose other microbial patterns to be recognized. Thus, whether through soluble or cell-anchored means of recognizing microbial-specific components, pattern recognition serves a critical role in innate immune recognition.

DANGER SENSING

While ligands for the PRRs are clear signals of danger to the immune system, there are other important means by which dangerous conditions can be promptly defined by the host. Specifically, receptor systems are in place to recognize and respond to physiological ligands whose expression is reserved for dangerous circumstances. The exposure of these ligands, therefore, acts as a surrogate marker for the presence of danger. If enough ligand is recognized, a critical signal amplification threshold can be achieved to induce a response. There are two broad categories of danger sensing: soluble immune mediators and danger ligands/receptors.

There are a wide variety of soluble immune mediators that, when secreted, can signify danger and induce a protective response. These factors are able to contribute to innate immunity, are encoded in the germline DNA, and are induced under specific stressful conditions. As an example, some are produced by cells that become infected. The purpose of this is to allow for innate immune defenses in the cells of the local environment to respond against the imminent danger. One example is type-I interferon. An infected cell will produce type-I interferons, leading to the induction of antiviral innate defenses in local cells that recognize the type-I interferon via specific receptors. The recognition of type-I interferon induces proteins such as protein kinase R (PKR) and Mx proteins, which slow down the proliferative capacity of the cell and impart specific antiviral properties. PKR inhibits translation by phosphorylation of the transcription factor eIF-2, while Mx proteins are GTPases that prevent nucleocapsid transport and viral nucleic acid replication. Thus, by recognizing a host-encoded soluble signal for danger, an innate immune defense can be initiated.

There are also a number of receptor–ligand pairs encoded in the germline DNA that are specifically accessed in the context of danger. These represent an inherent distress call that a cell can put out in situations of stress to facilitate its recognition as needing to be contained or eradicated. An example is the expression of CD48 on B cells infected by Epstein-Barr virus (EBV). This ligand was originally named Blast-1 for its appearance on EBV-transformed B-cell lymphoblasts. CD48 serves as a ligand for CD244 (2B4), which is a useful activating receptor constitutively expressed on NK cells. Its ability to sense danger through the recognition of CD48 represents an endogenous sensing mechanism that is immediately available to access innate immunity due to the constitutive expression of CD244. Another similar example is that certain infected or malignant cells that will express MICA as a feature of the stress these processes induce. MICA is specifically recognized by the constitutively expressed NKG2D receptor present on NK and T cells and like CD244 can directly induce innate immunity. Thus, while using endogenous receptor–ligand pairs, danger-sensing mechanisms can interpret abnormalities to effectively participate as elements of innate immune recognition.

AMPLIFICATION PHASE OF INNATE IMMUNITY

The recognition phase of innate immunity is central to the identification of danger, but in most cases does not directly include function that is relevant to host defense. To enable an effector response, the recognition of a danger signature needs to be amplified. This typically includes amplification events inside the cell recognizing the initial danger, as well as subsequent communication to the tissues and other components of innate immunity. Thus the amplification phase can be intracellular, extracellular, or both.

INTRACELLULAR AMPLIFICATION

Intracellular amplification follows the recognition of a danger signature by an innate immune receptor, or after the recognition of specific host-derived ligands by a danger-sensing receptor. The amplification typically involves a series of adaptor molecules and signaling effectors such as kinases, phosphatases, and ubiquitin ligases. At least some of these molecules are

recruited to the signaling platform centered around the receptor that has been ligated in order to amplify a signal generated after the recognition. The variety of intracellular signaling pathways and paradigms used by innate immune receptors is extensive and usually specific to a particular receptor system. As there is great diversity of these signaling mechanisms, they will only be illustrated here using the TLR system, which is well defined at the molecular level. There are, however, superb independent reviews of intracellular amplification resulting directly from the recognition of danger (Akira and Takeda, 2004; Beutler, 2009; O'Neill, 2008; Takeda et al., 2003).

The TLR system has been extensively studied for the signaling events that are utilized during intracellular amplification and has been reviewed elsewhere. The intracellular amplification following ligation of TLR is also relevant to consider, since defects in some of the molecules involved have been identified as causing specific primary immunodeficiency diseases (Picard et al., 2003; von Bernuth et al., 2008) (see Chapters 34–36). The TLRs are not entirely overlapping with regard to the amplification events that occur after their ligation (Fig. 8.1). They all, however, share the primary objective of inducing transcriptional machinery to lead to the production of new proteins. These newly synthesized proteins can then act to further amplify the innate response, or to serve roles as innate immune effectors.

The specific amplification pathways used by the TLRs, however, do have some important similarities. Amplification signals resulting from the ligation of all TLRs require the signaling adaptor MyD88 to access transcriptional functions. The exceptions to this rule are TLR3 and 4, which can amplify a signal in a MyD88-independent manner via the use of the TRIF and TRAM adaptor proteins. This MyD88-independent pathway then utilizes TBK to induce further downstream events. Ultimately all of the different amplification pathways will lead to accessing a kinase or kinase complex that induces the activation/phosphorylation of a transcription factor. For TLR this is most notably NF-κB (Fig. 8.1A) but includes others such as AP-1, IRF3, 5, and 7 (Fig. 8.1B). NF-κB is a transcription factor critical for innate immunity that is induced after dimers are released from the hold of the inhibitor of NF-κB (IκB) in the cytoplasm so that they can translocate to the nucleus and promote transcription. IκB is targeted for proteosomal degradation by ubiquitinylation, after it is phosphorylated by the IκB kinase (IKK) consisting of IKKα/β and the NF-κB essential modulator (NEMO). Most TLRs are capable of activating NF-κB in an IRAK4- and TRAF6-dependent manner. TLR3 can also function to activate NF-κB but uses RIP1 instead of IRAK4, and TRAF6. A number of other transcription factors are also induced through TLR signaling via distinct TLR-induced kinase functions. These include the AP1 transcription factor via MAPK by most TLRs; the IRF3 transcription factor via TBK1 by TLR4 and TLR3 (independently of MyD88); the IRF5 transcription factor via TRAF6; and the IRF7 transcription factor via IRAK1 specifically by endosomal TLR.

The transcription factors activated downstream in the process of TLR signal amplification further serve to amplify signals by inducing the generation of new proteins. Some of these proteins serve important intracellular functions and include components of transcription factors themselves (such as IRF and NF-κB), apoptosis regulators, and antigen-processing machinery. Other genes transcribed by TLR-induced transcription factors lead to the production of proteins that serve important roles in extracellular amplifications of the innate immune response and are discussed below. The TLR-induced transcription factors, however, also induce the production of effector molecules that are essential to the response phase of innate immunity and are discussed in the next section.

EXTRACELLULAR AMPLIFICATION

The amplification phase of innate immunity can be initiated extracellularly or it can have extracellular components after an intracellular amplification has occurred. Extracellular amplification can be through soluble mediators and thus lead to the involvement of innate immune elements from a distance. An example of a soluble amplification that is not initiated intracellularly is the fixation and activation of complement (discussed in greater detail in the specific section on innate immune components) (also see Chapter 55). Complement activation has the capacity to generate the chemotactic anaphylatoxins C3a, C4a, and C5a, which recruit other innate immune elements for action. Another example of a soluble amplification is the generation of proinflammatory cytokines and chemokines that are released from a cell after an intracellular amplification following a receptor-based recognition of danger. This example is germane to the theme of TLR as NF-κB activation after TLR ligation will lead to the transcription, translation, and release of soluble mediators, such as the potent chemoattractant chemokine CXCL10 (Kawai et al., 2001; Re and Strominger, 2001). These in turn will induce the activation and attraction of other innate immune cells so that they can participate in the defense.

Extracellular amplification can also occur through cell–cell contacts. In this case, the cell recognizing danger can express other activating ligands, such as co-stimulatory molecules or surface-anchored cytokines. These can then amplify the signal to a neighboring cell, which will then begin the process of intracellular amplification. An example would be that dendritic cells responding to TLR ligation induce cell-surface IL-15, which in turn leads to intracellular amplification in and activation of NK cells (Lucas et al., 2007). Alternatively, the initial innate immune cell can secrete factors in a directed manner onto a neighboring cell to enable its activation specifically. An example is a dendritic cell secreting IL-12 directly onto an NK cell, which in turn amplifies that signal to secrete IFN-γ onto the dendritic cell (Borg et al., 2004). This leads to a locally amplified signal that is now ready to initiate an innate immune effector mechanism.

RESPONSE PHASE OF INNATE IMMUNITY

The response phase of innate immunity is characterized by effector functions that are directly linked to the recognition of danger and pursuant signal amplification. These include (1)

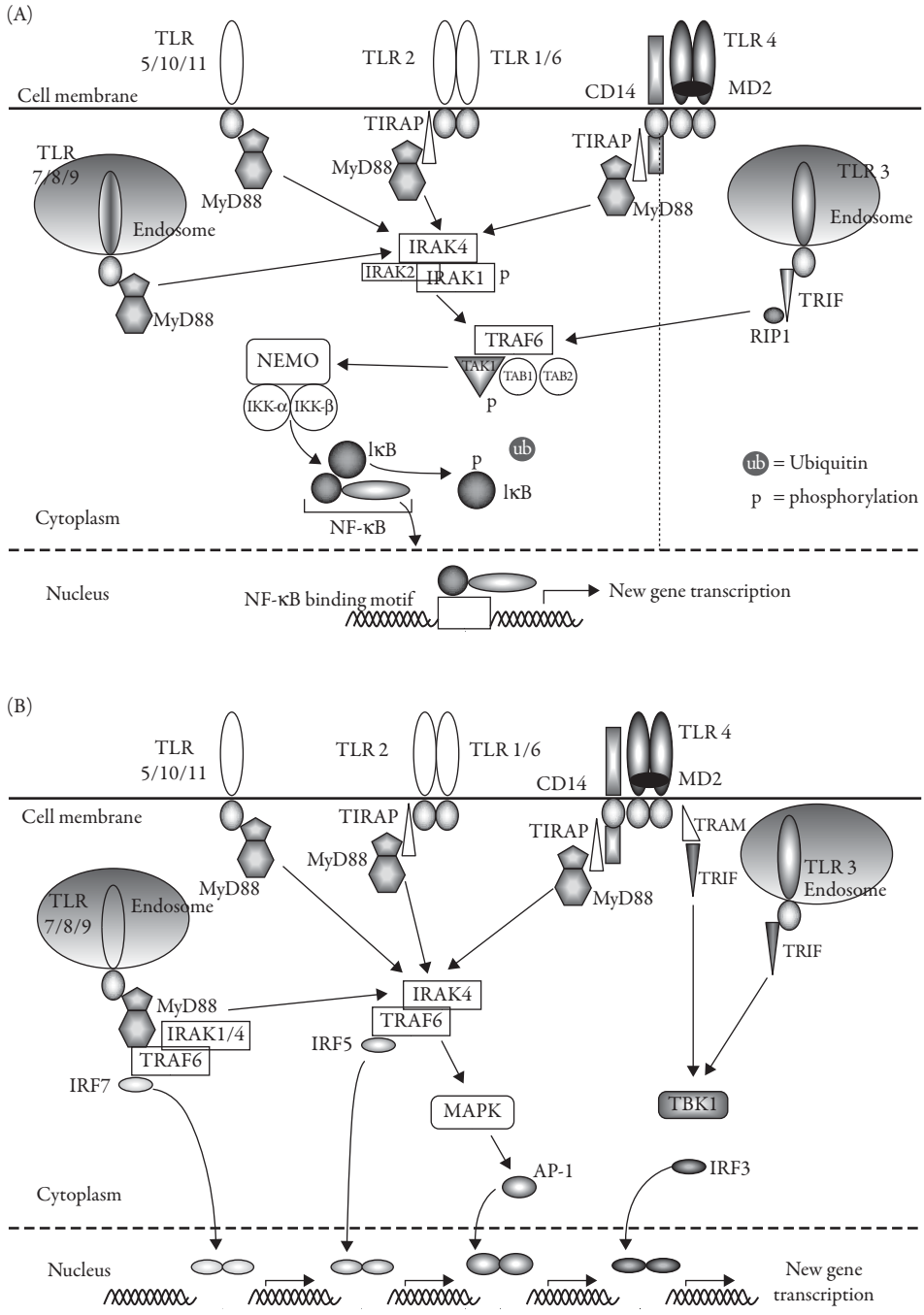

Figure 8.1 *The intracellular amplification phase of innate immunity as demonstrated by TLR signaling.* Ligation of the cell surface or endosomal TLR can lead to induction of NF-κB function (A) or of an assortment of other transcription factors (B). In either case specific adaptor proteins downstream of the TLR are utilized to access intracellular kinases to ultimately activate the transcription factors. These diagrams are not intended to offer an exhaustive scheme of signaling downstream of TLR, but to summarize some of the major routes that signaling can take to induce transcriptional activation. Each independent protein component is shown with a separate labeled shape. The solid horizontal line at the top denotes the cell-membrane boundary between the cell surface and the extracellular environment, and the dashed horizontal line at the bottom specifies the nuclear envelope. The dashed vertical line notes the separation between the MyD88-dependent and -independent signaling pathways. Importantly, TLR4 can amplify a signal in either a MyD88-dependent or -independent manner. With NF-κB activation the combination of NEMO, IKKα, and IKKβ represents the IKK complex, which phosphorylates IκB. This leads to its dissociation from NF-κB as well as targeting it for ubiquitination and proteosomal degradation (not pictured). The horizontal coil in the nucleus represents DNA and the rectangle depicts a promoter sequence in the DNA (not a protein) capable of binding the given transcription factor.

effects upon the physiology and local tissues preparing them for the battle against the pathogen or danger (i.e., inflammation); (2) direct innate effector mechanisms; and (3) communication with adaptive immunity.

LOCAL EFFECTS

Inflammation represents a means of priming the local environment for promoting antimicrobial responses and is one effector mechanism inherent to many innate immune pathways.

The characteristics of inflammation include increased local blood flow, increased vascular permeability, and activation of endothelium to facilitate chemoattraction of other cellular components of innate immunity. Inflammation occurs in response to the release of numerous soluble innate immune mediators, including prostaglandins, leukotrienes, TNF, IL-1, IL-6, and IL-12. To continue with the example of the TLR system, TNF, IL-2, and IL-6 are all produced as a result of the transcriptional activation following TLR ligand recognition. Thus, recognition of a danger motif can directly enable inflammation at the site of recognition.

DIRECT INNATE EFFECTORS

The innate immune system also possesses numerous important direct effector mechanisms that can participate in controlling or containing infection without the need for other factors. Some of these include the soluble effector proteins of the innate immune system such as antimicrobial peptides, and complement. These can mediate the direct destruction of a microbe and thus have an inherent and direct effector function (discussed in greater detail below). Some of these, including several antimicrobial peptides, are synthesized within and secreted by cells of the innate immune system.

The cells of innate immunity can possess their own effector mechanisms, some of which are shared by the adaptive immune system. Mechanisms that are shared with adaptive immunity include perforin-dependent cytotoxicity, contact-dependent cytotoxicity mediated by TRAIL, FAS-L, and surface-bound TNF. Effector mechanisms, which are specialties of certain innate immune cells, include phagocytosis and generation of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). Phagocytosis generally occurs after an organism is opsonized by complement, or antibody, or ligates a specific PRR. The ingested organism is then destroyed by ROIs, RNIs, acidification of the endosome, or iron restriction via proteins such as natural resistance-associated macrophage protein 1 (Nramp1). ROIs are generated from the NADPH oxidase (phox) complex that can reduce oxygen to superoxide by adding an electron (Fig. 8.2). Superoxide dismutase and myeloperoxidase can then generate hydrogen peroxide and hypochlorous acid (see Chapter 52). RNIs are generated when nitric oxide (NO) synthases (NOS) catalyze L-Arg + oxygen to form L-citrulline and NO. There are three forms of NOS: NOS1 and NOS3 are constitutively expressed and NOS2 is induced.

Although the above-mentioned systems represent only a few examples of effector mechanisms, they illustrate the breadth of means by which the innate immune system can directly mediate function against microbes. While some of these mechanisms are clearly sufficient in their own right to eliminate a challenge, they can also be useful in limiting the spread of a dangerous challenge until the adaptive immune system can be recruited to participate in the response.

LINKS TO ADAPTIVE IMMUNITY

A third and essential response of innate immunity is in its ability to link to the adaptive immune system (Beutler, 2004).

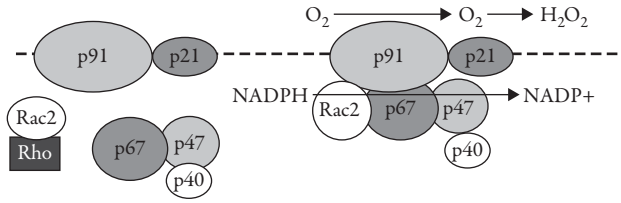

Figure 8.2 *The phagosome oxidase (phox) complex: an essential example of the response phase of innate immunity.* The phox complex consists of numerous components depicted by individual shapes. After recognition and amplification has occurred, the components can assemble to form a functional complex, as represented by the transition from the left to the right of the figure. The dashed line represents the phagosomal membrane. Two components, p91 and p21, are integral to the membrane (left), and the others join the complex at the site of the phagosome after activation has occurred (right). For assembly to occur, Rac2 must become free of Rho, which results as a part of the signal amplification. When assembled, the phox complex reduces NADPH to generate superoxide from oxygen. Using cellular superoxide dismutase, the generated superoxide can be converted to hydrogen peroxide, which has potent antimicrobial function (see Chapter 52 for details).

Through a variety of effector mechanisms, innate immune cells and mediators can constructively communicate with cells of the adaptive immune system. This can serve the purpose of helping to initiate or induce an adaptive immune response, which in turn can allow the adaptive immune system to more specifically target the dangerous challenge. The adaptive immune response can then potentially minimize any collateral damage that the innate immune system may have been responsible for while containing the challenge. Although this topic is rather broad, it is essential to appreciate its existence and relevance. A few important examples are provided.

The innate immune system can produce cytokines that are able to promote the development of adaptive immune cells into functionally specialized cell subsets. Although this has a variety of applications, the classical example is that of polarization of T-helper-cell subsets. If IL-12 is produced by the innate immune system when T cells are developing, they can be biased toward Th1 and away from Th2 differentiation. Clearly, this has important consequences on participation of the adaptive immune system in host defense.

A clear and critical function of innate immunity in interfacing with adaptive immune response is in antigen presentation. The "professional" antigen-presenting cell of the immune system is the dendritic cell (DC), which is an integral part of innate immunity. DCs, described in greater detail in a specific section below, are capable of presenting antigenic peptides to T cells. They perform this function by processing proteins and loading peptides into major histocompatibility molecules (MHC), which are trafficked to the cell surface, where they can be recognized by T cells. Importantly, the antigen-presentation process in DC is facilitated by recognition of TLR ligands and subsequent signal amplification (Blander and Medzhitov, 2006).

Innate immunity is also an essential source of chemokines and chemoattractive factors that can enable the adhesion of and recruit adaptive immune cells to critical sites where antigen can be provided. An example mechanistically defined in

mice is that of NK cells producing the chemokine CCL22 to recruit T cells to tumor microenvironments (Mailloux and Young, 2009).

There are also other important roles for contact-dependent effector functions of innate immunity in linking to the adaptive immune system. An example is the number of co-stimulatory ligands expressed by innate immune cells that provide critical signals to the adaptive cells. An example is the expression of CD86 (B7.2) by DCs after they have been activated. To follow the theme introduced earlier, CD86 can be induced on DCs after they have recognized patterns via TLRs and amplified signal (Hertz et al., 2001). Once expressed, CD86 is recognized by CD28 expressed on T cells, thus generating the essential second signal for T-cell proliferation that is needed in addition to ligation of the T-cell receptor. Thus, interaction between the innate and adaptive immune system is essential for host defense and represents a critical response of innate immunity.

COMPONENTS OF INNATE IMMUNITY

In addition to considering the specific paradigms with innate immunity functions, it is useful to be cognizant of the individual components that make up the innate immune system (Beutler, 2004). On a rudimentary level these can be divided into three categories: physical barriers; proteins and soluble mediators; and cells. Each is introduced below with specific reference to important members of the specific category.

PHYSICAL BARRIERS AND INNATE IMMUNITY

In a sense physical barriers can be viewed as a form of innate immunity. While the majority of cells and tissues that form such barriers are not of immune origin, they do constitute a critical defense against the environment and are essential in protection against infection and other danger. Thus they are at least worthy of mention in the context of innate immunity. Important physical barriers include the cornified epithelium of skin, intestinal epithelium, as well as less well-formed barriers such as mucous linings of various tissues. In addition, the cells of the innate immune system do contribute directly to these barriers in many cases by secreting peptides, enzymes, and other factors to augment the inherent defense functions of physical barriers. An example would be the secretion of defensins (discussed below) into mucus that coats the airway epithelium.

SOLUBLE AND SECRETED COMPONENTS OF INNATE IMMUNITY

Many soluble and secreted molecules are critical components of the recognition, amplification, and effector functions of the innate immune system. Some of these function primarily in amplification and can be considered as mediators, while others possess direct recognition and/or effector functions. Both of these groups of molecules within the innate immune system are extensive, and thus a few key illustrations of soluble and secreted molecules/proteins are given to introduce the major categories.

COMPLEMENT

Complement is a collective term introduced 100 years ago to define a group of factors present in fresh plasma that when activated by specific antibody were able to kill bacteria (Wagner et al., 1998). Later work showed the bacteria studied were lysed and that the killing principle was heat-labile—that is, heated normal serum lost this killing ability. We have come to recognize that this collective term includes at least 30 known proteins and protein regulators, some of which circulate in the blood and some of which are bound to cell membranes. These proteins play a major role in host defense and innate immunity (see Chapter 55). More recently, it has become clear that complement is important also in the generation of a normal immune response. Phylogenetically, the complement proteins are ancient, serving a host defense function in animals as primitive as sea urchins. The adaptive immune system appears in evolution at the level of the jawed fish and, by this point in evolution, all the various complement proteins are arrayed to produce their regulatory and host defense functions. The system clearly follows the pattern of pathogen recognition, amplification, and response.

Three pathways of complement activation are known (Fig. 8.3) (Walport, 2001a, 2001b). The first pathway, defined over a century ago, and therefore termed the classical pathway, appears in phylogeny at the time of antibody and usually uses antibody as its recognition molecule. A second pathway, termed the alternative pathway because it was recognized later, is phylogenetically the oldest pathway and does not require antibody for pathogen recognition. Its recognition mechanism, although complex, is quite primitive and will be described below. The third pathway, the lectin pathway, described in the past two decades, is still being defined in detail (Jensenius, 2005). Its recognition molecules are lectins, mannose binding lectin and the group of ficolins, proteins that recognize repetitive sugar motifs. In this case the sugars are found as part of the capsules or envelopes of most bacterial and viral pathogens and, as such, constitute pathogen-associated molecular patterns. Because normal human proteins and cells do not have repeating sugar patterns, these lectins, which bind to the sugars expressed on the surface of pathogens by multiple low-affinity sugar-recognition sites and activate complement, tend not to bind to host cells. All three pathways proceed by engaging a related series of serine protease enzymes to the activation site and by binding and activating a plasma protein, C3, present at high plasma concentration (1.2 mg/mL), that is central to all three pathways. Upon activation of C3, a fragment, C3a, is cleaved from the molecule and the remaining large fragment of C3, C3b, has a metastable binding site generated that can bind covalently to any nearby cell, bacterium, or virus or can be hydrolyzed by water. Bound C3b can interact with other alternative pathway proteins to initiate further activation of

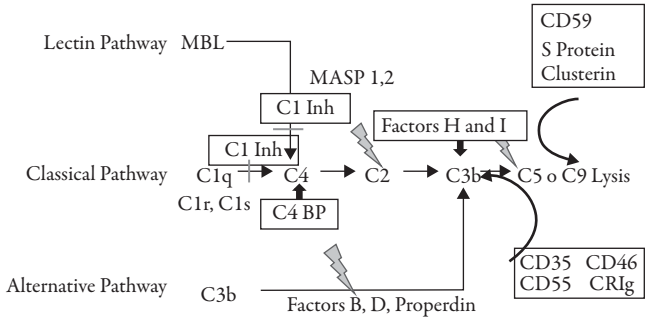

Figure 8.3 *Schematic of complement pathways.* Complement control proteins are listed in boxes. Those proteins that are membrane bound are in bold print; those that are fluid phase are not bolded. The lightning bolts direct attention to those proteins that bind to the assembling complement complex so that an unstable site is formed that decays with loss of ability to continue the cascade. C1 inhibitor controls activation of the classical and lectin pathway. C4 binding protein controls the biological activity of the active fragment of C4, C4b. Factors H and I are the major fluid-phase controllers of C3b. C3b binds preferentially to CD35. Factor H can bind to C3b, allowing it to be cleaved by factor I. Cleavage of the α chain leads to the formation of hemolytically inactive C3b (iC3b). This molecular form of C3 binds preferentially to CD11b/CD18 or CD11c/CD18. CD35, CD46, and CD55 control further aspects of the degradation and cleavage of C3. CRIg is a macrophage C3b receptor found prominently on Kupffer cells in the liver that facilitates removal and phagocytosis of C3b-coated particles from the circulation and aids in the degradation of C3b. CD59 prevents assembly of the membrane attack complex, as do the fluid-phase proteins S protein and clusterin.

the alternative pathway, thus amplifying its effect. If bound to a pathogen surface, C3b and its degradation products can initiate the steps leading to pathogen destruction. C3 undergoes spontaneous activation in the blood, and it is C3 itself that acts to initiate the alternative pathway. Since activated C3 can also bind to host cell membranes, there are proteins that recognize C3b deposited on a host cell surface and cause it to be destroyed without destroying the C3b on a microorganism surface. Nevertheless, it should be clear that more sophisticated mechanisms for pathogen recognition have evolved to facilitate an individual's ability to fight off infection. Because space precludes a discussion of the function of each of the proteins in the various complement pathways, Figure 8.3 illustrates their name designation and place in the various activation pathways. After binding C3, the pathways proceed together through the binding of an additional series of proteins, C5, C6, C7, C8, and C9, to promote the lytic and inflammatory steps in complement action.

There are three major effector functions of complement in host defense. First is its ability to lyse cells. The binding of C3 and the later binding of C5 to C9 leads to the formation of a cylinder-like structure that inserts itself into the surface of complement-sensitive cells, bacteria, or enveloped viruses. The cell or organism cannot maintain its internal stability as its internal environment is in free communication with the external milieu and it dies. The second important function of complement is to opsonize particles. On complement activation, complement fragments, particularly those produced by activation of C3, may bind covalently to an organism surface. These can interact with specific receptors for these fragments on the surface of cells like neutrophils and macrophages, facilitating the ingestion process. The third essential effector function of complement is the ability to generate on activation cleavage fragments that have potent inflammatory activity. For example, the small fragment of C5, C5a, can cause mast cells to degranulate and release histamine as if they were coated with IgE and antigen. C5a can cause migration of phagocytic cells toward the place where the peptide is generated—that is, induce chemotaxis—and it can cause cytokine release from cells (Hugli, 1986; Nordahl et al., 2004).

The complement activation steps are enzymatic and complement activation will continue until the system is specifically turned off. Thus, the system is built to provide massive amplification of small signals. Because activation of complement can induce unwanted widespread inflammatory tissue damage, the system is under tight regulatory control. There are both fluid-phase and cell membrane-bound proteins that function to downregulate or inhibit complement function. Although absence of a complement protein because of a gene abnormality is a very rare event in the case of most of the complement proteins, absence of one of the regulators is more common (see Chapter 55). Such absence, even when the absence is partial, may lead to impressive disease. So, for example, when one of the two gene alleles for factor H, a proteins that in part helps degrade C3b deposited on a host cell surface, is absent, the patient may develop a variant of hemolytic-uremic syndrome with destruction of his or her own kidneys as well as autoimmune erythrocyte hemolysis.

MANNOSE-BINDING LECTIN (MBL)

MBL is an example of the collectin family of calcium-dependent lectins and will bind to terminal mannose groups on many bacteria (Jensenius, 2005). Complement is activated via the MBL-associated serine proteases (MASPs). MASP1 can cleave C3 directly and MASP2 cleaves C4 and activated C2. Thus, MBL represents an additional demonstration of innate recognition, which leads to amplification via complement and effector function via an assembled complement MAC (See Chapter 55). It has been found to have diverse roles in innate immunity (Ip et al., 2009), and animals deficient in MBL are susceptible to staphylococcal infection (Shi et al., 2004). The isolated role of MBL in human host defense, however, is the subject of substantial study and is at present unclear (Casanova and Abel, 2004).

ANTIMICROBIAL PEPTIDES

Endogenous soluble secreted antimicrobials are increasingly appreciated as an effector component of innate immunity. The family of antimicrobial peptides comprises cationic proteins ranging in size from 8 to 45 amino acids in their fully processed forms, which can insert into concentrations of bacterial phospholipids to slow microbial growth (Agerberth and Gudmundsson, 2006). Many of these are synergistic or additive in this action. They include the defensins, cathelicidins, lactoferrins, and histatins.

Defensins are triple-stranded molecules held together by three disulphide bonds. The defensin family includes α-defensin or human neutrophil peptide (HNP), and β-defensin or human β-defensin (hBD). The HNP subfamily consists of HNP1–4 expressed by granulocytes and HNP5 expressed in the intestine. The HBD subfamily consists of hBD1–3 expressed in skin and lung, and hBD-4 expressed in testis. Of particular potential relevance to human disease mechanisms, hBD-2 is decreased in atopic dermatitis relative to levels found in psoriasis lesions or normal skin (Ong et al., 2002).

Cathelicidins are distinguished by possessing a cathelin-like domain at their N-terminus and an antimicrobial domain at their C-terminus. An important member of this subfamily is LL-37, which is expressed by granulocytes and in lung. LL-37 is also found to be decreased in lesions of active atopic dermatitis relative to levels found in psoriasis lesions or normal skin (Ong et al., 2002).

Lactoferrins belong to the transferrin family of proteins and under normal circumstances bind iron. The human lactoferrin (hLF) subfamily consists of hLF1–11, which can be produced by macrophages, among other cells. Importantly, they are also found in tears and in human milk.

Histatins are distinguished by having histadine-rich sequences, and the subfamily contains at least 12 members. The histatins are abundant in saliva and salivary glands and as a result probably serve an important role in defense against microbes entering the oral cavity. They are especially active against fungi.

PROTEOLYTIC ENZYMES

The innate immune system secretes an important variety of proteolytic enzymes that can have critical roles in host defense and the direct elimination of danger. The granzymes are a central example and are serine proteases contained within the lytic granules of cytotoxic cells. When pores are created in pathogens or dangerous cells via molecules such as perforin, granzyme entry and uptake can occur. In humans the granzyme family includes granzymes A, B, H, K, and M, each of which has particular patterns of expression. The cathepsin family also represents an important group of proteases, but these are cysteine as opposed to serine proteases. In addition to roles in host defense, cathepsins serve important functions in antigen-presenting cells to further process entocytosed proteins.

CYTOKINES AND CHEMOKINES

Cytokines and chemokines are a large group of soluble protein messengers that share the common characteristic of having action upon immune cells. They can be produced by immune as well as by nonimmune cells and are characterized by a finite family of structures. They are the subject of many superb reviews that are focusing on nomenclature, origin, and function (Borish and Steinke, 2003; Ransohoff, 2009; Rossi and Zlotnik, 2000; Steinke and Borish, 2006). In some cases these factors can function as cell-surface molecules, but more commonly they are secreted to function in soluble formats in local or distant environments. In either case, they can participate in amplification and response phases of innate immunity, and some specific examples were mentioned above in the section on paradigms in innate immunity.

Some critical innate members of the cytokine and chemokine families are worth specific emphasis. Among the cytokines, these include the members of the proinflammatory cascade, including IL-12, TNF, IL-1, IL-6, and IL-18. These factors are rapidly induced and secreted upon the recognition of danger and serve essential roles in preparing cells and tissues to handle threats as well as further activate and induce immune responses. Together these factors represent a very powerful attempt of the innate immune system to mobilize a response and can be contained on a local level, but can also be pronounced systemically in situations of grave danger. In the most extreme circumstances this can precipitate a "systemic inflammatory response syndrome" in which the host is risking survival with a most aggressive response in order to gain control over danger (Castellheim et al., 2009).

Chemokines are generally more specialized in the recruitment of additional elements of immunity. They can be produced by the innate immune system to recruit additional innate elements, or components of the adaptive immune system. An example of the innate system recruiting more innate immune elements is CXCL8 (also known as IL-8), which is produced by activated macrophages, NK cells, and epithelium (Remick, 2005). It can ligate its major receptor CXCR1 on neutrophils and induce their chemotaxis toward the source of CXCL8. To follow the theme introduced earlier, CXCL8 secretion can be potently induced by ligation of TLR4 by LPS (Sabroe et al., 2003), thus demonstrating an essential role for IL-8 in the amplification phase of innate immunity. An example of the innate immune system specifically recruiting the adaptive immune system is CCL20 (Schutyser et al., 2003). The production of CCL20 is highly induced by ligation of TLRs (Scapini et al., 2001) and facilitates the recruitment of T cells through the CCR6 receptor to the sites of amplification where adaptive immune responses can be propagated.

NEUROPEPTIDES

Neuropeptides are a family of small proteins that by definition are produced by cells of the nervous system. In many cases, however, they can also be produced by cells of the innate immune system and as a group can have numerous and important effects on immune function. Examples include adrenomedullin, proenkephalin A, and neurokinin-1 (also known as substance P). Some of these peptides, such as neurokinin-1, also possess direct antimicrobial functions and thus have significant overlapping function with the antimicrobial peptides discussed above (Brogden et al., 2005). Neurokinin-1 can be produced by and also serve to potently stimulate or inhibit innate immune functions and as such is important to consider as an alternative regulator of innate immunity (Tuluc et al., 2009).

CELLS OF INNATE IMMUNITY

Most cells are capable of innate responses to danger, as having the ability to recognize a challenge to the host is critical

to their survival. Many cells express and can utilize the same pattern recognition, amplification, and response systems discussed above. As an example, TLRs are widely expressed both inside and outside the immune system. They can facilitate the recognition of danger in innate immune cells, but also in cells of the adaptive immune system and in nonimmune cells such as fibroblasts. Thus, innate recognition of danger serves important roles in many contexts. There are, however, cells that should be considered as specialized or "professional" cells of the innate immune system. For the purposes of this discussion they can be considered to include neutrophils, monocytes/macrophages, DCs, eosinophils/mast cells/basophils, and NK cells and are summarized in Table 8.2.

NEUTROPHILS

Neutrophils, also referred to as polymorphonuclear leukocytes, are the chief phagocytes of the immune system (see Chapters 50–53). They develop from $CD34^+$ hematopoietic stem cells in the bone marrow in response to IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). The later developmental stages of neutrophils, including transition to a granulocyte precursor from one that has the potential to develop into either a monocyte or granulocyte, is especially dependent upon G-CSF. Neutrophils represent more than half of the hematopoietic cells that develop from bone marrow and are relatively short-lived in the circulation ($t_{1/2}$ ~6 hours) as well as in the tissues ($t_{1/2}$ ~24 hours). They are distinguished by cell-surface expression of CD67 (CD66b), also known as carcinoembryonic antigen-related cell adhesion molecule 8, which may be involved in neutrophil aggregation. Neutrophils also express receptors aimed at facilitating their major objective of phagocytosis. These include Fc receptors, and the complement receptors CR1 and CR3 to facilitate phagocytosis through the binding of opsonizing antibody and complement, respectively.

Neutrophils serve essential roles in host defense through their ability to engulf and destroy pathogens (Segal, 2005). They are attracted to sites of danger via an array of chemotactic mechanisms, including the above-mentioned fMLP receptor and CXCL8. They also are potently recruited by C5a, CCL3 (MIP-1α), platelet activating factor, and leukotriene B4, among others. They adhere to their target site using the well-defined mechanism of rolling and tight adhesion dependent upon cell-surface–expressed selectins and integrins, respectively. The destruction of engulfed pathogens can occur via oxygen-dependent (Fig. 8.2) or oxygen-independent systems. The oxygen-independent mechanisms can involve many of the different effector molecules discussed above and include proteins contained in neutrophil granules.

Neutorophil granules represent specifically compartmentalized effector units that are maintained for use in host defense. Their separation from the remainder of the cell allows for the ready storage of highly toxic substances without harming the normal functions of the cell. There are four major types of neutrophil granules, azurophil, specific, gelatinase, and secretory, each having specialized roles. The azurophil granules contain largely host defense molecules, including lysozyme, defensins, acid phosphatase, glucosidases, cathepsins, and elastase. These organelles can contribute to the antimicrobial effect against phagocytosed organisms (contained in a phagosome) by joining the phagosome once it has been formed. The azurophil granule then contributes its contents to the phagosome to provide additional effector mechanisms in the attempted destruction of the pathogen. Neutrophil-specific granules contain the fMLP receptor, integrins, lysozyme, procollagenase, B12 binding protein,

Table 8.2 **THE "PROFESSIONAL" CELLS OF INNATE IMMUNITY**

CELL	KEY FUNCTION	DISTINGUISHING CELL-SURFACE MARKERS	HALF-LIFE	DISTINGUISHING FEATURES	DEVELOPMENT	CHEMOTAXIS
Neutrophils	Phagocytosis	CD67	~6 h (circulation) ~1 d (tissues)	Contain 4 granule types—azurophil, specific, gelatinase, secretory	IL-3, GM-CSF, G-CSF	fMLP, CCL8, CCL3
Monocytes/Macrophages	Phagocytosis, secretion, antigen presentation	CD11b CD14 CD36	~70 h (circulation) Years (tissues)	Potently activated by IFN-γ	GM-CSF M-CSF	CCL4, CCL22, CXCL4
Dendritic cells	Antigen presentation	MHC-II CD11c (most)	~1 d to 2 wks	4 major subsets: myeloid, plasmacytoid, Langerhans, interstitial	GM-CSF IL-4	CCL3 CCL21
Eosinophils/Mast cells/Basophils	IgE-binding, effector secretion	FcεRI (M,B), CD23 (E)	~6 h (E, B) ~21 d (M)	Respond to IgE	SCF (M) IL-3 (B) IL-5 (E)	CCL5 CCL11 (E)
NK cells	Cytotoxicity, secretion	CD56, CD158 CD335 CD337	~10d	Lytic granules containing perforin and granzymes	IL-15	CXCL12

and lactoferrins. By contributing these molecules the specific granule can augment neutrophil chemotaxis, adhesion, and host defense. Their importance is underscored as they are missing in the primary immunodeficiency specific granule deficiency, resulting from mutation of the CCAAT/enhancer-binding protein ε transcriptional regulator (Lekstrom-Himes et al., 1999). Neutrophil gelatinase granules have similarities to specific granules and also contain the fMLP receptor, integrins, and lysozyme, but are distinguished by their gelatinase content. The gelatinase granules are also distinguished by their rapid mobilization after presumed TLR4 ligation by LPS (Almkvist et al., 2001). Neutrophil secretory granules contain many of the same molecules as the specific granules, including those used in chemotaxis and adhesion, but are distinguished by their expression of alkaline phosphatase. Neutrophil alkaline phosphatase is a phosphatidylinositol-linked transmembrane receptor that functions to facilitate dephosphorylation in alkaline environments.

MONOCYTES/MACROPHAGES

Monocytes and macrophages have many overlapping functions with neutrophils as they are active in both phagocytosis and secretion, but also overlap with DCs as they can function in antigen presentation (Auffray et al., 2009; Serbina et al., 2008). Importantly, monocytes are much longer-lived than neutrophils, with a half-life of 70 hours in the circulation. When marginating to tissues, monocytes have the unique characteristic of transitioning into macrophages and can persist for years. This can facilitate tissue-specific immunity but has proven important in disease pathogenesis by serving as long-term reservoirs for the human immunodeficiency virus (Alexaki et al., 2008). Monocytes originate from the granulocyte-macrophage colony-forming unit (GM-CFU) in the bone marrow and require M-CSF and GM-CSF for development. From the circulation, monocytes are attracted to tissues via a number of factors, including CCL4, CCL22, and CXCL4. In tissues, macrophages are capable of performing phagocytosis and generating reactive metabolites similarly to neutrophils. They also secrete over 100 different functionally relevant proteins, including TNF and other proinflammatory cytokines. Monocytes are very potently activated by IFN-γ, which from an innate immune standpoint is derived from NK cells, but of course is also derived from many cells of the adaptive immune system. Distinguishing cell-surface receptors expressed on the monocyte/macrophage include CD11b/CD18 (Mac1), which is also expressed on neutrophils and NK cells; CD14, which is an LPS co-receptor; and CD36, which is an LDL scavenger important for recognizing apoptotic cells. As elements of host defense and promoting immunity, monocytes/macrophages are instrumental, but pure human deficiencies of this lineage have not been defined, except, perhaps, the recently described monocytopenia and mycobacterial infection (MonoMAC) syndrome due to mutations in GATA2. However, MonoMAC patients also have profound NK and B-cell lymphopenia and absent DCs in the circulation, findings that may contribute to the immunodeficiency associated with this syndrome (Hsu et al., 2011).

DENDRITIC CELLS

DCs can be considered as part of the innate immune system, although they perhaps represent the critical link between innate and adaptive immunity. DCs, best known for their function as "professional" antigen-presentation cells, are specialized for presenting peptides in the context of class II MHC to T cells. They facilitate the activation of T cells when antigen is presented along with co-stimulation but can induce anergy when co-stimulation is withheld (Novak and Bieber, 2008). These co-stimulatory molecules include B7.1 (CD80) and B7.2 (CD86), which interact with CD28 on T cells. Importantly, this co-stimulation-dependent link between innate and adaptive immunity can be induced through the innate function of DCs in recognizing danger. Specifically, ligation of TLR on DCs can lead to their upregulation of CD80 and CD86 (Gaddis et al., 2009; Krug et al., 2001). In this sense DCs are innate gatekeepers of the adaptive immune response and depend upon danger sensing to regulate their communication. In addition, the specific conditions of their differentiation and microenvironment can help them preferentially drive T cells to have a polarized functional phenotype, such as to T-helper 1 or 2 cells. DCs additionally interface with the adaptive immune system by their role in the germinal center reaction in the lymphoid follicle required for the generation of an antibody response (Randolph et al., 2008).

DCs develop from bone marrow hematopoietic stem cells facilitated by GM-CSF and IL-4. They are considered immature when resting in tissues and have a high capacity for antigen uptake. DCs are considered mature after activation and have decreased capacity for antigen uptake but increased MHC expression. In this state they migrate to lymphoid organs to facilitate the interface with the adaptive response and can be recruited by CCL3 and CCL21, among others. There are four major DC subsets, myeloid, plasmacytoid, Langerhans, and interstitial, although some focus divisions of DCs more specifically upon myeloid and plasmacytoid.

Myeloid DCs (also known as DC1) develop from a common myeloid progenitor and express characteristic myeloid markers, including CD11b/c, CD13, and CD14. CD13 on DCs likely serves a useful role in reducing the length of peptide presented in class II MHC to facilitate antigen presentation (Larsen et al., 1996). They also express multiple CD1 isoforms to enable alternative antigens to be presented. Myeloid DCs are efficient in phagocytosis, but less so in producing type I interferon.

Plasmacytoid DCs (also known as DC2 and lymphoid DCs) originate from the common lymphoid progenitor and express some characteristic lymphoid markers, including CD4, and the Igλ-like 14.1. Plasmacytoid DCs can produce high levels of type I interferons, which are important in initiating antiviral responses.

Langerhans DCs develop from cutaneous lymphocyte antigen (CLA)-expressing $CD34^+$ precursors originating from common myeloid progenitor cells. They express CD11c and langerin (CD207) and have characteristic Birbeck granules. Langerin functions in the uptake of microbial components as

well as inflammatory cell recruitment. Birbeck granules are tennis racquet-shaped membrane-associated organelles that can be distinguished by electron microscopy. Langerhans cells are often found in the epidermis, excel in $CD8^+$ T-cell priming, and are the longest-lived of the DC subsets.

Interstitial DCs (IDC) develop from CLA^- $CD34^+$ precursors derived from common myeloid progenitor cells. Unlike Langerhans DCs, IDCs express abundant CD2, CD9, and CD68. They do not possess Birbeck granules, do not express CD207, and are found in the dermis. They are effective in macropinocytosis, IL-10 production, and inducing B-cell activation.

EOSINOPHILS, MAST CELLS, AND BASOPHILS

While certainly representative of distinct and important functional units, eosinophils, mast cells, and basophils are discussed collectively due to their common roles in atopic pathogenesis (Blanchard and Rothenberg, 2009; Prussin and Metcalfe, 2003, 2006; Rothenberg and Hogan, 2006). Although atopic diseases present a great burden to the developed world, the major role and likely evolutionary purpose of these cells is in host defense against large extracellular parasitic pathogens such as helminths (Galli et al., 2002). All of these cells have the ability to respond to IgE, which is a characteristic part of the anthelminthic immune response. Basophils and mast cells recognize IgE through the high-affinity receptor (FcεRI) and eosinophils through a lower-affinity receptor (CD23). Each of these cell types is capable of releasing large numbers of soluble mediators that can contribute to amplification and directly to response phases of innate immunity. Important examples of the latter include eosinophil expression of major basic protein (MBP) and eosinophil cationic protein (ECP). Both of these can be secreted and specifically enable the effective participation of eosinophils in host defense (Gleich et al., 1979; Yazdanbakhsh et al., 1987). In particular, both MPB and ECP alter the permeability of membranes and can promote the dissolution of the target; this is especially effective against helminths. Together with basophils, eosinophils also have an important role in resistance against certain ticks (Brown et al., 1982). Mast cells are defined by their tissue localization and are divided into two major subsets based upon whether they contain tryptase, or tryptase and chymase. When secreted in response to IgE in tissues, these components can have a direct ability to exert antimicrobial effects (McNeil et al., 2007), in addition to actively promoting inflammation.

NATURAL KILLER CELLS

NK cells are lymphocytes derived from the common lymphoid progenitor in the bone marrow that require IL-15 for their development (Orange and Ballas, 2006). They have a number of characteristic cell-surface markers, including CD16 (a low-affinity Fc receptor), CD56 (neural cell adhesion molecule), the natural cytotoxicity receptor (NCR—CD335, CD336, CD337) family of molecules, and killer cell immunoglobulin receptors (KIR—CD158 and others). They do not express CD3 and unlike most other lymphocytes do not rearrange their TCR or Ig genes. NK cells are activated by a number of receptors that recognize danger ligands, such as NKG2D recognizing MICA (Raulet, 2003). They also can be specifically induced by their own set of pattern-recognition receptors, such as NKp46 recognizing viral hemagglutinins (Gazit et al., 2006; Mandelboim et al., 2001). NK cells can additionally be activated by the Fc of IgG through their Fc receptor and thus can recognize opsonized materials.

NK cells are inhibited by a number of receptors that recognize characteristics of self, such as the KIR family that recognizes class I MHC. The absence of self-molecules in a target cell, such as in tumor or virus-infected cells, enables NK cell function and has given rise to what is known as the "missing self hypothesis." In combination with the presence of ligands for activation receptors in these target cells, downmodulation of self-MHC can enable NK cell function. Thus, activation and function in NK cells is a feature of a balance favoring activation signaling (Culley et al., 2009; Lanier, 2008).

There are 14 KIR genes in humans on chromosome 19. The genes are highly polymorphic, and there are a large number of distinct KIR haplotypes that can be inherited. As experience with KIR molecules and genotypes has grown, one feature that has become apparent is that like MHC, there are definitive links between the KIR haplotypes and human disease (Parham, 2005). An important example is the presence of KIR2DL3 in the haplotype resulting in rapid clearance of hepatitis C virus when the infected individual is homozygous for the HLA-C1 ligand (Khakoo et al., 2004).

Once activated, NK cells can have three major functions: cytotoxicity, cytokine production, and co-stimulation. NK cytotoxicity is contact-dependent and thus is similar to that employed by cytotoxic T cells. It utilizes perforin and granzymes that are contained within lytic granules. Triggering the NK cell with an appropriate activation receptor results in the intracellular traffic of the lytic granules toward the target cell upon which they are extruded (Orange, 2008). NK cell cytotoxicity is important in eliminating virus-infected cells, especially those infected with herpesviruses as well as hematopoietic tumor cells that have lost expression of class I MHC. In bone marrow transplantation for malignancy, developing donor NK cell cytotoxicity cells is likely responsible for significant graft antitumor effect (Ruggeri et al., 2002). This requires a mismatch between the specificity of the inhibitory receptors present on the donor cells to the class I MHC ligands present in the recipient.

In addition to functioning in cytotoxicity, NK cells are highly efficient in producing IFN-γ and do so to participate in antiviral defense (Orange et al., 1995). NK cell IFN-γ is also very important in facilitating macrophage and DC functions to improve innate immunity. NK cell IFN-γ additionally can directly augment adaptive immune cells. This function overlaps with the ability of NK cells to efficiently provide contact-dependent co-stimulation and can do so through their expression of CD252 (OX40/CD134L) and CD154 (CD40L). Thus, while diverse in their functions, NK cells

are the major lymphocytes of the innate immune system and serve important roles in defense. This is most clearly illustrated by a number of rare human deficiencies of NK cells (Orange, 2006, 2011).

CONCLUSIONS

Innate immunity represents the initial line of defense against danger. There are three phases in innate immunity—recognition, amplification, and response. Each has important elements that relate not only to the generation of host defense, but also to consideration of immunodeficiency. Recognition is focused upon the rapid identification of danger, either the recognition of molecular patterns or by specific danger-sensing mechanisms. TLRs are a major example within innate immunity of pattern recognition and have been used throughout this chapter to illustrate the paradigms of innate immunity. Similar concepts, however, apply to the numerous other systems we did not have opportunity to address in detail. The amplification phase of innate immunity can occur intracellularly via signaling pathways or through extracellular mechanisms. Finally, the response phase of innate immunity is characterized by the specific production of soluble effector molecules, contact-dependent effects, and promoting adaptive immunity. While there are many individual components of innate immunity, it is useful to consider these paradigms when evaluating potential defects of innate immunity that impair host defense (see Chapters 34–36).

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9.

INTRODUCTION TO SEVERE COMBINED IMMUNODEFICIENCY (SCID) AND COMBINED IMMUNODEFICIENCY (CID)

Jennifer M. Puck

BRIEF HISTORY OF SCID

The first descriptions of a fatal congenital deficiency of lymphocytes date from the 1950s in Switzerland. Glanzmann and Riniker (1950) described an idiopathic wasting syndrome with fatal *Candida albicans* infection. Hitzig and Willi (1961), following the discovery of agammaglobulinemia by Bruton (1952), reported familial alymphocytosis combined with agammaglobulinemia with a fatal outcome in infancy. The term "Swiss-type agammaglobulinemia" was originally used to distinguish infants with fungal infections, lymphopenia, and early death from the less severely affected children who came to medical attention somewhat later in life with isolated agammaglobulinemia. However, the term was a source of confusion, in part because the "Swiss-type" label was then applied to kindreds in which SCID was inherited as an X-linked recessive trait. "Nezeloff syndrome" was another term originally describing cases of thymus dysplasia with preservation of immunoglobulins (Gosseye et al., 1983; Nezelof, 1968), but the term was no longer used after it became clear that B-cell immunity could not develop in the absence of T cells. In subsequent decades SCID designations have become more precise thanks to improved immunological tools to characterize immune defects plus the identification immunodeficiency genes.

Failure to recognize immunodeficiency as the underlying cause of recurrent diarrhea, pneumonia, septicemia, fungal infections, or failure to thrive is evident in family histories of kindreds in which affected infants have died. In the past and even today many SCID patients have been mistakenly thought to have dietary intolerances or cystic fibrosis because of diarrhea, poor weight gain, and pulmonary infections. Some have received diagnoses such as scarlet fever from the rash of spontaneous graft-versus-host disease from maternally derived lymphocytes; others have been thought to have diphtheria when thrush extending down the throat resembled a diphtheritic membrane. In countries where newborns are routinely vaccinated against tuberculosis with the live attenuated mycobacterial organism bacillus Calmette-Guérin (BCG), infants with SCID may develop fatal disseminated BCG infection, and inadvertent administration of attenuated live poliovirus vaccine, and more recently live rotavirus vaccine, to infants with SCID has led to paralytic polio and severe diarrheal disease, respectively (Bakare et al., 2010). Unfortunately, SCID is frequently not suspected upon presentation to primary physicians or even referral centers; this has spurred recently successful efforts to institute newborn screening for SCID (Chan et al., 2011; Kwan et al., 2013; also see Chapter 57).

Basic and clinical research in SCID has led to fundamental advances in our understanding of lymphocyte development as well as pioneering medical treatments extending far beyond this rare collection of inherited defects. Prior to 1968, patients with SCID died in infancy. However, the pioneering immune reconstitution following bone marrow transplantation (BMT) from an HLA-identical sibling marked the beginning of successful treatment (Gatti et al., 1968; see Chapter 59). The lack of a histocompatible sibling donor led to the use of isolation in a germ-free environment for "David the Bubble Boy," a famous Texas patient who came to represent SCID to the general public. Although David succumbed to complications of newly recognized Epstein-Barr virus lymphoproliferative disease after a BMT at age 12 (Shearer et al., 1985), techniques for transplantation with T cell-depleted haploidentical bone marrow from a parent and with unrelated adult or cord blood hematopoietic cells from well-matched donors have made SCID treatable for all patients (Bortin and Rimm, 1977; also see Chapter 60). Adenosine deaminase (ADA)-deficient SCID was the first disease treated with enzyme therapy (see Chapter 14), and ADA and X-linked

SCID pioneered successful human gene therapy (Cavazzana-Calvo et al., 2000; see Chapter 60). Most recently, the first population-based screening for congenital immune disease was established for SCID (Chan and Puck, 2005; Routes et al., 2009; also see Chapter 57).

Single gene mutations that result in SCID have revealed proteins and pathways that are nonredundant (i.e., essential) for the development and function of human adaptive immunity. Mouse knockouts of the same genes do not reliably produce identical phenotypes, underlying the importance of studying immune defects in humans. While seven genes—*IL2RG*, *IL7R*, *JAK3*, *ADA*, *RAG1*, *RAG2*, and Artemis (*DCLRE1C*)—account for around 90% of SCID cases, many additional genes, even beyond those in Table 1.1 in Chapter 1, continue to be discovered, enriching our understanding of the complex system that provides humans with the ability to recognize and overcome infections throughout life.

LYMPHOCYTE PROFILES AND DIVERSE GENE DEFECTS IN SCID

Table 1.1 lists SCID genotypes and their associated cellular phenotypes. Enumeration of T, B, and natural killer (NK) lymphocytes provides useful correlations of lymphocyte phenotype with genotype (Buckley et al., 2004; Kalman et al., 2004; Stephan et al., 1993), although there are well-documented exceptions (Puck et al., 1997). While all patients with SCID have very few autologous T cells, over half have detectable or even increased numbers of B cells that fail to produce specific antibodies (T^-B^+ SCID). Most of these patients also lack NK cells and are males with X-linked *IL2RG* defects (Noguchi et al., 1993; Puck et al., 1993; see Chapter 10). However, mutations in autosomal *JAK3*, encoding the signaling kinase that interacts intracellularly with the common γ chain, also cause $T^-B^+NK^-$ SCID (Russell et al., 1995; see Chapter 10). Other T^-B^+ SCID genes are *IL7R*, encoding the α chain of the receptor for IL-7 (Puel et al., 1998; see Chapter 10); *CD45*, encoding the cell-surface co-receptor CD45 (Kung et al., 2000; see Chapter 12); and genes encoding components of the CD3 complex (see Chapter 11). In addition, humans with defects in the *FOXN1* transcription factor that is defective in athymic nude mice have autosomal recessive T^-B^+ SCID in addition to alopecia totalis (Frank et al., 1999; see Chapter 21).

SCID with no T or B cells (T^-B^- SCID) occurs in an even ratio of males to females due to autosomal recessive inheritance and is most commonly caused by ADA deficiency (Giblett et al., 1972; see Chapter 14) and also by defects in the proteins involved in the DNA rearrangement of T-cell and B-cell receptor genes, most notably recombinase activating genes *RAG1* and *RAG2* along with the Artemis or *DCLRE1C* gene (Chapter 13).

COMBINED IMMUNODEFICIENCY

Primary immunodeficiency conditions in which T-cell immunity is impaired but not completely absent and B cells are also impaired (due either to intrinsic defects or lack of helper functions supplied by normal T cells) constitute CID. Some patients with CID have "leaky" defects in the same genes in which null mutations produce typical SCID (Table 1.1). A small amount of protein production from these genes, or protein with reduced function, gives rise to a spectrum of phenotypes including susceptibility to infections as well as dysregulated immunity. Disease presentation with infections may occur later in life, or with more mild illness compared to infants with typical SCID, but also distinct syndromes are recognized, including Omenn syndrome, defined as erythroderma rash, eosinophilia, and oligoclonal autologous T cells that cause adenopathy and organ infiltration (Omenn, 1965). A combination of hypomorphic mutations in *RAG1* or *RAG2* genes with overcompensation and dysregulation of other immune reactions can lead to combined T and B cell immunodeficiency with granulomatous disease (Schuetz et al., 2008).

Additional cases of CID are due to null mutations in "variant" SCID genes that generally have a less devastating impact on T-cell production than do typical SCID genes (Puck, 2012); these are also listed in Table 1.1. In these conditions, including Zap70 deficiency, MHC class I or class II deficiency, and others with known and as yet unknown molecular defects, T cells may be produced from the thymus in normal or near-normal numbers, but are impaired in function. In addition, several congenital syndromes with multiorgan involvement such as cartilage-hair hypoplasia, multiple intestinal atresias or CHARGE (ocular coloboma, heart defect, atresia of nasal choanae, retardation of growth and development, genitourinary abnormality, and ear abnormality) syndrome have variable degrees of T- and B-cell immunity, which can be severe.

MAKING THE DIAGNOSIS OF XSCID

A helpful clue from a simple laboratory test is a low absolute lymphocyte count, compared to values for age-matched normal individuals (Shearer et al., 2003; Buckley, 2004). Once the clinical suspicion of an immunodeficiency has been raised, whether by family history, low lymphocyte count, or recurrent or opportunistic infections, the diagnosis of SCID or CID must be pursued by lymphocyte phenotyping by flow cytometry after staining with monoclonal antibodies. Lymphocyte phenotyping in infants can be misleading in the face of maternal T-cell engraftment, transfusion of nonirradiated blood products, certain infections, or expanded populations of oligoclonal cells with poor function. Fewer than 300/μL naïve autologous T cells in peripheral blood strongly suggests SCID. Functional analysis of lymphocytes, such as with proliferation to the mitogen phytohemagglutinin (PHA) is essential to distinguish secondary causes of low T cells from primary SCID diseases, in which proliferation is generally less than 10% of the lower limit of normal for the test. Documentation of a pathogenic mutation by gene sequence should also be sought; increasingly, differences in responses to specific treatments are being recognized dependent on genotype so that molecular diagnosis assists in the selection of an optimal treatment regimen.

Leaky SCID and variant SCID do not demonstrate maternal engraftment and generally have 300 to 1,500 T cells, though oligoclonal T-cell expansion can be associated with higher numbers of memory phenotype cells; nonetheless, responses to mitogens such as PHA and other functional assessments show impairment compared to normal T cells. There are still SCID and CID patients whose molecular diagnosis remains unknown, and additional disease genes are sure to be found.

Because of defective development of T cells, all infants with SCID and many with CID lack T-cell receptor excision circles, or TRECs (Chan and Puck, 2005). TRECs are DNA circles formed by end-joining of genomic DNA segments excised during normal T-cell receptor gene rearrangement; a quantitative PCR reaction across the joined ends is used to measure TREC number (Douek et al., 2000). TREC detection in newborn dried blood spots has formed the basis of population-based newborn screening, now used in a growing number of states in the United States (Chan and Puck, 2005; Puck, 2012; Kwan, 2013; also see Chapter 57). Identification of presymptomatic cases of T lymphocytopenias necessitated development of new classifications and laboratory-based diagnostic criteria because they do not manifest infectious complications of immunodeficiency at birth. Infants with low or undetectable TRECs in dried blood spots and confirmatory flow cytometry evaluations indicating low T-cell numbers may have typical SCID, leaky or variant forms of SCID, syndromes that include low T cells as part of their phenotype—all discussed above—or alternatively may have secondary lymphocytopenia associated with other medical conditions, including vascular permeability, congenital heart defects, extreme prematurity, or other conditions. Whether primary or secondary, these patients should avoid exposure to live vaccines or unirradiated or CMV-positive transfusions and need to be followed by immunologists until their T cells normalize or until definitive therapy for their immunodeficiency is accomplished.

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10.

SEVERE COMBINED IMMUNODEFICIENCY AND COMBINED IMMUNODEFICIENCY DUE TO CYTOKINE SIGNALING DEFECTS (*IL2RG*, *JAK3*, *IL7R*, *IL2RA*, *JAK3* AND *STAT5B*)

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INTRODUCTION

Severe combined immunodeficiency (SCID) and combined immunodeficiency (CID) are the terms applied to a collection of genetic defects that involve both humoral and cellular immunity. All types of SICD and CID are characterized by impaired generation of a diverse repertoire of mature T lymphocytes, and many have additional immune abnormalities. The profound lack of immune function in SCID leads to infections that are generally fatal in infancy unless the immune system can be reconstituted. CID is characterized by somewhat less profound abnormalities and therefore may present somewhat later in life. These are rare disorders, whose precise incidence is unknown because until recently presentation most often occurred with serious infections secondary to the immunodeficiency; therefore, infants who died of infections before a primary immune defect was suspected were not counted (Chan et al., 2011). Now that newborn screening is available for SCID (see below and Chapter 57), the impaired production of T cells can be detected apart from infectious complications (reviewed in Buckley, 2012; Puck, 2012; Verbsky et al., 2012). Over the coming years the new paradigm of presymptomatic diagnosis will no doubt change many aspects concerning the conception, confirmation, treatment, and outcomes for individuals with SCID and CID.

Immunophenotypic and functional analysis of circulating lymphoid cells permits an accessible and useful classification of SCID into distinct subgroups, as presented in Table 1.1 (Fischer, 2001): reticular dysgenesis and adenosine deaminase (ADA) deficiency, generally lacking T, B, and NK cells (T^- B^- NK^-); defects of T- and B-cell antigen receptor recombination (T^- B^- NK^+); defects with low T and NK cells, but normal to increased B cells (T^- B^+ NK^-); and defects predominantly or exclusively restricted to T cells (T^- B^+ NK^+). The subgroups of SCID with B lymphocytes (B^+ SCID) account for over 50 percent of all cases of SCID (Antoine et al., 2003; Bertrand et al., 1999; Buckley, 2000a, Buckley, 2000b, 2004; Fischer et al., 1990; Haddad et al., 1998; Stephan et al., 1993). Most are due to known molecular defects that interfere with signaling of a series of critical hematopoietic cytokines.

DISCOVERY OF THE X-LINKED SCID DISEASE GENE, *IL2RG*

Both a striking male predominance of SCID in all published series and recognition of pedigrees with multiple affected males related through maternal lineages long suggested that X-linked SCID (XSCID, SCIDX1, MIM#300400, MIM*308380) would turn out to be the major form of SCID, and indeed this was the first SCID gene defect to be identified after ADA deficiency, which causes T^- B^- NK^- SCID (see Chapter 14). XSCID was first mapped by linkage to the proximal long arm of the X chromosome by De Saint Basile et al. (1987). Gene mapping studies were hampered by the early lethality of XSCID in males and the lack of any clinical or immunological abnormalities to distinguish carrier from non-carrier females. However, Puck et al. (1987) noted that in contrast to the expected random X chromosome inactivation in female tissues predicted by the Lyon hypothesis, obligate female carriers of XSCID had only the nonmutated X chromosome as the active X in their lymphocytes. This nonrandom X inactivation reflected a selective disadvantage in proliferation, differentiation, or survival of lymphocyte progenitors lacking a normal gene product at the SCIDX1 locus. Nonrandom X inactivation was found in T, B, and NK cells of XSCID carriers. This indirect evidence, 6 years before the underlying molecular defect for XSCID

Table 10.1 CLINICAL FEATURES OF TYPICAL X-LINKED, JAK3-DEFICIENT AND IL7RA-DEFICIENT SEVERE COMBINED IMMUNODEFICIENCY NOT DIAGNOSED BY FAMILY HISTORY OR NEWBORN SCREENING*

Universal Features
Male sex in X-linked SCID; both male and female infants may have JAK3- or IL7rα-deficient SCID
Presentation in first year, usually by age 6 months
Failure to thrive
Oral thrush, candida diaper rash
Absent tonsils
Persistence of infections despite conventional treatment
Common Features
Chronic diarrhea
Respiratory congestion, cough
Fevers
Pneumonia, especially with *Pneumocystis jiroveci*
Sepsis, severe bacterial infections
Viral infections, including cytomegalovirus, adenovirus, Epstein-Barr virus, enteric viruses, varicella, herpes and respiratory syncitial virus
Infections with opportunistic pathogens
No palpable peripheral lymph nodes
Family history of similarly affected maternal male relatives
Less Common Features
Erythematous skin rash (often with hepatomegaly and lymphadenopathy) from spontaneous or transfusion-related graft-versus-host disease
Recurrent bacterial meningitis
Disseminated infections (Salmonella, varicella, vaccine strain [live] rotavirus diarrhea, disseminated Bacillus Calmette-Guérin infection; rarely, vaccine strain [live] polio with paralysis)

*Affected infants who are diagnosed prior to onset of infectious complications (whether by population-based newborn screening or early diagnosis due to a positive family history—see Chapter 57) appear healthy but lack tonsils and lymph nodes.

was identified, showed that the B cells of patients affected with XSCID were intrinsically defective and not merely lacking T cell help. In contrast, random X inactivation patterns were seen in their granulocytes, monocytes, and other tissues (Conley et al., 1988; Puck et al., 1987; Wengler et al., 1993). Thus, the SCIDX1 gene product is necessary for the development and survival of lymphoid but not myeloid cells from bone marrow stem cells.

Nonrandom X inactivation in lymphocytes was used to assign the carrier status of female relatives of XSCID patients, increasing the number of informative individuals for genetic linkage in XSCID pedigrees. Further narrowing of the SCIDX1 region was made possible by the study of males with the eye malformation choroideremia in combination with deafness, but not immunodeficiency, who had interstitial deletions below Xq13 (Puck et al., 1993a). Until this time, no genes related to the immune system were known to reside in the region defined by these mapping studies. However, in 1993, two groups (Noguchi et al., 1993a; Puck et al., 1993b) realized that the γ chain of the receptor for IL-2, cloned the previous year (Takeshita et al., 1992), was located on the X chromosome in the SCIDX1 critical region. Deleterious mutations in *IL2RG*, the gene encoding this receptor, were then found by both groups in XSCID patients, proving that *IL2RG* is the XSCID disease gene.

Only males, who have a single X chromosome, are affected with XSCID. Female carriers, who have a mutated copy of the gene on one and a normal copy on the other of their two X chromosomes, remain healthy. However, carrier females can pass on the disease to their sons and the carrier state to their daughters. When the XSCID genetic locus in Xq13.1 was identified so that mutation diagnosis could be performed, a wide variety of defects were demonstrated (Buckley et al., 1997; Puck et al., 1997a; Niemela et al., 2003; Kellermayer et al., 2006; Lee et al., 2011). XSCID accounts for close to half of all SCID cases in series published from transplant centers, though perhaps a larger proportion in the United States than in European populations (Antoine et al., 2003; Buckley, 2004; Buckley et al., 1997). As newborn screening reveals the incidence of different SCID genotypes in the future, without the bias of clinical detection and referral to transplant centers, the proportion of XSCID may be updated. XSCID appears to occur equally in all ethnic groups.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF SCID

Male infants with XSCID, and males and females with other cytokine pathway SCID defects, appear normal at birth. Unless

Table 10.2 PATHOLOGICAL FINDINGS IN TYPICAL X-LINKED, JAK3-DEFICIENT AND IL7Rα-DEFICIENT SEVERE COMBINED IMMUNODEFICIENCY

Primary absence of T-cell precursors
Markedly small thymus gland
Vestigial thymic stroma present
Absence of lymphocytes in thymus
No Hassall's corpuscles in thymus
No corticomedullary distinction
Evidence of infections secondary to immunodeficiency
Candida pharyngitis, esophagitis
Pneumonia or pulmonary infiltration with:
***Candida*, other fungi**
***Pseudomonas*, other gram-negative bacteria**
Pneumocystis jiroreci
Cytomegalovirus
Evidence of graft-versus-host disease
Lymphocytic infiltration of skin, liver, other organs

preinfectious diagnosis is made either by population-based newborn screening or by testing in the setting of a positive family history, presenting complaints reflect infectious complications and can be highly variable, as summarized in Table 10.2. These features are generally not distinguishable from patients with autosomal recessive (AR) forms of SCID except that female patients are assumed to have AR SCID. Although a family history can lead to laboratory confirmation of SCID before or at the time of birth, over 80 percent of patients have no recognized relatives with diagnosed immunodeficiency or early death consistent with SCID (Chan et al., 2011). Whether due to chance and small sibship sizes or to newly arising mutations, most SCID cases of any genotype are sporadic (Buckley et al., 1997; Conley et al., 1990).

As transplacentally transferred maternal IgG wanes, infants with SCID develop infections that bring them to medical attention, generally by 3 to 6 months of age. Thrush or respiratory and gastrointestinal infections may at first seem routine, but they do not respond to the usual medical management. For example, otitis may not resolve despite several courses of oral antibiotics; respiratory syncytial virus (RSV) may be continuously present in pulmonary secretions for several months; and diarrhea that may follow community exposure or inadvertent administration of live attenuated rotavirus vaccine (Patel et al., 2010) leads to failure to gain weight, or even weight loss. Rashes, either erythematous or maculopapular, have been associated with graft-versus-host disease (GVHD), either from maternal cells transferred to the infant during birth or from transfusion of nonirradiated blood products prior to recognition of the immunodeficiency.

Eventually, a clinical decompensation, diagnosis of an opportunistic pathogen, such as *Pneumocystis jiroveci* (formerly *P. carinii*), or a high index of suspicion leads to consideration of an immune disorder. Decompensating events include bacterial sepsis, meningitis, or deep-seated infections, as well as bacterial, fungal, or viral pneumonias. Series of SCID patients in France (Stephan et al., 1993) and the United States (Buckley et al., 1997) reported a similar large range of infectious agents at presentation: *Pseudomonas*, *Salmonella,* and other gram-negative bacteria; *Streptococcus*, *Staphylococcus* and other gram-positive bacteria; respiratory viruses and viruses of the herpes family; and fungi, primarily *Candida albicans* and *P. Jiroreci*. Infants with SCID who have inadvertently received BCG vaccination may develop disseminated infection with this organism. Live polio vaccination has also caused poliomyelitis and carditis, but this complication is uncommon, probably because most infants have protective transplacentally acquired maternal anti-poliovirus antibodies. Rotavirus live attenuated vaccine can cause diarrhea in infants with SCID.

The hallmark of immunopathology (Table 10.2) is an extremely small thymus gland almost devoid of lymphocytes. Thymic stroma is present but not well differentiated. There are no Hassall's corpuscles and no corticomedullary distinction. Thymic dendritic and epithelial cells are abnormal. Analysis of T-cell receptor (TCR) β chain rearrangement in thymic tissue from infants with XSCID has shown that the initial Dβ-to-Jβ recombination can occur, but subsequent VDJ rearrangement is blocked (Sleasman et al., 1994). Similarly, in extrathymic lymphoid organs, lymph nodes are small and poorly developed, and T lymphocytes cannot be found. Tonsils are absent. Other pathological findings in infants with SCID who do not survive include manifestations of their terminal infections, and in some patients, lymphocytic infiltration of skin, liver, and other organs as a result of GVHD.

Unless treated by hematopoietic cell transplantation (HSC) or experimental gene therapy, infants with SCID generally succumb to infections before 1 year of age, but in rare cases the diagnosis has been made in infants beyond their second birthday. A sheltered home environment may be a factor in late presentation.

LABORATORY FINDINGS IN SCID

Laboratory values that are useful to diagnose all types of SCID are summarized in Table 10.3. A helpful clue from a simple laboratory test is a low absolute lymphocyte count, compared to values for age-matched normal infants. While not universal, this indicator should raise the suspicion of a congenital immunodeficiency in an infant with a significant infection. Because of arrested development of T cells, infants with SCID lack TCR excision circles, or TRECs (Chan and Puck, 2005). TRECs are DNA circles formed by end-joining of genomic DNA segments removed during TCR gene rearrangement; a quantitative PCR reaction across the joined ends is used to measure TREC number (Douek et al., 2000; Schönland et al., 2003). TREC measurement has been developed into a successful population-based method for screening newborns using the dried blood spots obtained routinely to test for a variety of inborn defects (reviewed in Buckley, 2012; Puck, 2012; Verbsky et al., 2012).

Table 10.3 LABORATORY ABNORMALITIES IN INFANTS WITH X-LINKED AND JAK3-DEFICIENT SEVERE COMBINED IMMUNODEFICIENCY*

	SCID PATIENTS	PROPORTION WITH LOW NUMBERS	CONTROL VALUE RANGE, 1ST YEAR*§
Lymphocyte Counts	<2,000	90%	3,400–9,000
Total lymphocytes/μL	200 (0–800, with higher values representing maternal cells)	100%	2,500–5,600
T cells/μL			
B cells/μL	1,300 (0–3,000)	5%	300–3,000
NK cells/μL	<100*	88%	170–1,100
T-cell receptor excision circles (TRECs)	Absent	100%	1 per 10 T cells
Antibody Concentrations§			
IgA, IgM	Extremely low		
IgG	Maternal levels at birth, low by age 3 months		
Lymphocyte Function§			
Mitogen responses	Very poor		
Mixed lymphocyte response	Very poor		
NK cytotoxicity	Usually very poor; rarely intact*		
Specific antibody	Very poor production		
Other Laboratory Tests			
Absent thymic shadow	Universal on chest radiogram		

*Findings are identical in IL-7-receptor-deficient SCID, except that NK cells are generally present in normal numbers and functional in this genotype.

§Control ranges are approximate and age-dependent, and they may vary between laboratories; consult the immunology laboratory performing the test for best interpretation.

Adapted from Buckley et al., 1993, 1997; Chan et al., 2004; Conley et al, 1990; Fischer, 1992; Kalman et al., 2004; Schearer et al., 2003; Schönland et al., 2003; Stephan et al., 1993; Stiehm et al., 2004.

Typically, but not always, the small numbers of lymphocytes found in patients with XSCID are predominantly or entirely B cells, which may be present in normal or even elevated numbers. The B cells in infants with XSCID are immature and resemble the naïve B-cell population of normal cord blood in their expression of cell-surface markers and in vitro antibody production restricted to IgM (Small et al., 1989). Sequence analysis of XSCID B-cell immunoglobulin heavy chains has demonstrated normal VDJ rearrangement (Minegishi et al., 1994). However, there is over-utilization of J_H3 segments, as seen in fetal and neonatal B cells, and a total lack of somatic hypermutation. In XSCID infants whose maternally acquired IgG antibodies have waned, antibody titers to vaccines or infectious agents are low or absent. Other distinguishing characteristics of XSCID are low numbers of cells that bear NK cell markers and poor NK cell cytotoxicity.

Despite the utility of these generalizations, lymphocyte numbers and function are not uniform in all patients and may be subject to modification by genetic factors other than *IL2RG* mutation and by environmental factors. For example, patients with *IL2RG* mutations occasionally have low rather than the typical normal to high numbers of B cells. Also, patients with proven *IL2RG* defects have been reported who have NK cells and intact NK cytotoxicity (Pepper et al., 1995).

Spontaneous engraftment of maternal lymphocytes can be detected in males with SCID by finding XX karyotypes on cytogenetic analysis, by HLA typing or by using DNA polymorphic markers. Maternal cells can be found in almost all SCID patients if sensitive methods are used, and if present in significant numbers they may resist engraftment of allogeneic HSCs. Although there may be no discernible consequences of maternal engraftment, the maternal cells may elevate the lymphocyte counts of an infant with SCID and display some proliferation to phytohemagglutinin (PHA). When sufficient numbers of maternal cells are activated to respond to the infant's paternally derived histocompatibility antigens, a SCID patient may have eosinophilia and circulating maternal, activated, DR^+ T cells. In patients who have iatrogenic GVHD from transfusion of nonirradiated blood products, allogeneic T cells can cause florid, acute GVHD, including gatroenteritis, hepatitis, and lymphocytic infiltrates in skin, liver, and other organs.

MOLECULAR BASIS OF XSCID: THE DISEASE GENE, *IL2RG*

IL2RG encodes the γ chain of the IL-2 receptor; the gene product appears in multiple receptors and is therefore now

called the common γ chain, γc. The α and β chains of the IL-2 receptor were known to exist before the γ chain was identified. The β chain, constitutively expressed on T cells, has intermediate affinity for IL-2; and the α chain, expressed after T-cell activation, boosts the affinity for IL-2 of the receptor complex 100-fold. The existence of a γ chain was postulated when transfection of the α and β chains of IL-2 receptor into fibroblast cell lines failed to produce a receptor that could transmit a signal. A third chain was co-immunoprecipitated with the β chain and purified by two-dimensional gel electrophoresis (Takeshita et al., 1992), permitting determination of the amino terminal sequence and isolation of the cDNA for *IL2RG*. The gene, depicted in Figure 10.1, spans 4.5 kb of genomic DNA in Xq13.1. The coding sequence of 1124 nucleotides is divided into 8 exons. *IL2RG* is one of the transmembrane cytokine receptor superfamily genes. It encodes a 5' signal sequence of 22 amino acids, which is cleaved off while targeting the protein for cell-surface expression. Four conserved cysteine residues at the extracellular amino-terminal end engage in cross-linking for proper conformation; the juxtamembrane extracellular motif WSXWS encoded in exon 5 is a hallmark of all cytokine receptors; the highly hydrophobic transmembrane domain of 29 amino acids occupies most of exon 6; and the proximal intracellular domain in exon 7 contains a Box1/Box2 signaling sequence homologous to SH2 subdomains of Src-related tyrosine kinases.

FUNCTION OF γC, THE *IL2RG* GENE PRODUCT

The first report of γc by Takeshita et al. (1992) showed that it was expressed in T- and B-cell lineages, but not liver or epithelial cells. Subsequent studies have shown expression in human and mouse hematopoietic progenitor cells throughout development of myeloid and lymphoid lineages (Orlic et al., 1997). Expression of γc on murine thymocytes increases as they mature from $CD4^-$ $CD8^-$ double-negative to double-positive and single-positive T cells, and further upregulation occurs after lymphocyte activation (Sugamura et al., 1996). Recognition that γc was a component of the IL-2 receptor explained why mitogen responses were poor in infants with XSCID. DiSanto et al. (1994b) showed absent binding and internalization of IL-2 in B-cell lines from XSCID patients. However, a mouse knockout of the gene for IL-2 manifested immune dysregulation with autoimmune hemolytic anemia and inflammatory bowel disease (Schimpl et al., 1994; Schorle et al., 1991). This apparent paradox may in part be explained if γc were to have additional roles. Indeed, several groups subsequently demonstrated it is part of the receptor complexes for multiple cytokines: IL-4 (Russell et al., 1993; Kondo et al., 1993), IL-7 (Noguchi et al., 1993b), IL-9 (Russell et al., 1994), IL-15 (Giri et al., 1994a), and IL-21 (Habib et al., 2002, 2003; Recher et al., 2011). IL-7 signals are important for moving early multipotent progenitors in the bone marrow into the lymphoid lineages; IL-4 participates in B-cell isotype switching (Kuhn et al., 1991; Matthews et al., 1995; Taylor et al., 1997); IL-15 signals are required for NK cell development (Kumaki et al., 1995; Matthews et al., 1995); and IL-21 signaling is especially important for augmenting T- and B-cell responses to antigens (Habib et al., 2003) and permitting normal B-cell differentiation (Recher et al., 2011).

MUTATION ANALYSIS IN XSCID

Several hundred *IL2RG* mutations have been reported in patients with XSCID from every racial group. A database

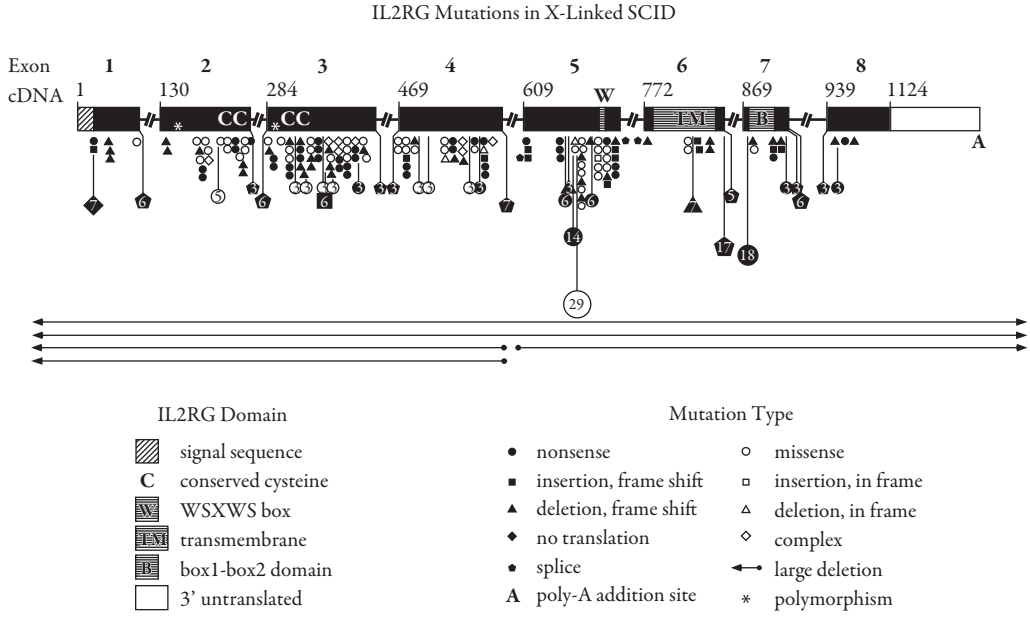

Figure 10.1 Structure of *IL2RG* and mutations found in patients with X-linked SCID and reported to IL2RGbase (http://www.research.nhgri.nih.gov/SCID/; Niemela et al, 2003; Puck et al., 1996); Genbank accession number L19546.

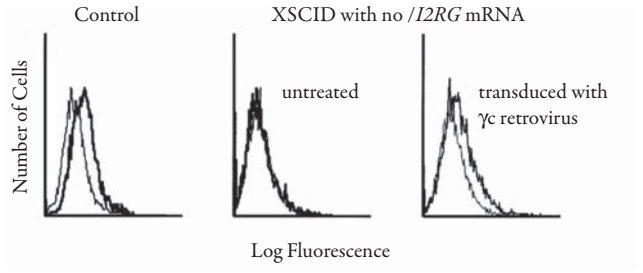

Figure 10.2 Expression of γc in B-cell lines from a healthy control (left), and from an XSCID patient with undetectable *IL2RG* mRNA, before (middle) and after (right) transduction with a retrovirus containing *IL2RG* cDNA under control of the Maloney leukemia virus LTR.

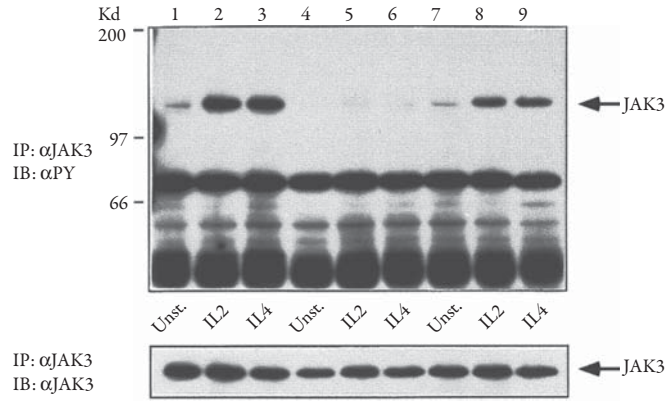

Figure 10.3 Phosphorylation of JAK3 induced by IL-2 and IL-4 in B-cell lines from a healthy control (lanes 1–3) and from an XSCID patient before (lanes 4–6) and after (lanes 7–9) transduction with an *IL2RG* retrovirus (Candotti et al., 1996). After no stimulation or exposure to IL-2 or IL-4, cell lysates were immunoprecipitated (IP) with anti-JAK3 antiserum (αJAK3) and immunoblotted (IB) with anti-phosphotyrosine (αPY), top, or αJAK3, bottom.

of published and verified mutations, IL2Rgbase (Puck et al., 1996), can be accessed at the Leiden Open Variation Database (http://www.lovd.nl/3.0/home). As indicated in Figure 10.1, and as is typical of SCID-causing mutations in other genes in addition to *IL2RG*, SCID-causing mutations occur throughout the entire length of the gene, including exons, splice sites, and even the 3' poly-A addition site. Recurrent mutations have been noted at several positions, or "hot spots," in *IL2RG*. In several instances, the new origin of a mutation has been proven by finding only the normal gene sequence in DNA from parents of female carriers or from mothers of affected males. Five hot spots for mutation involve the well-recognized mechanism of cytosine methylation and deamination to thymidine within a CpG dinucleotide. Almost all *IL2RG* mutations are changes in only one or a few nucleotides, but large deletions have occurred. The number and variety of mutations, including new mutations that continue to be found (Lebet et al., 2008), make gene sequencing imperative.

It is also important to assess the significance of missense mutations. Some are predicted to disrupt known essential elements (Fig. 10.1), such as the first methionine that signals initiation of translation, the four conserved extracellular cysteine residues, the WSEWS motif conserved in all members of the cytokine receptor gene family, or the hydrophobic transmembrane domain. Functional significance is likely for mutations at sites that are conserved not only among mammals, such as mouse, rat, and dog, but also between human and more distant species, such as birds or fish, or between *IL2RG* and other members of the cytokine gene family (Pepper et al., 1995). Amino acid substitutions repeatedly found to be the only change associated with XSCID in unrelated kindreds are more likely to be pathogenic than to be incidental polymorphisms. However, direct proof that a missense mutation is harmful requires testing a cell line from the affected patient for expression of *IL2RG* mRNA and determining whether γc protein is expressed at the cell surface (Fig. 10.2), whether expressed γc can bind IL-2, and whether downstream targets such as JAK3 and STAT5 become phosphorylated (Fig. 10.3) (Puck et al., 1993b, 1997a). Rare or private polymorphisms have been found in *IL2RG*, but none that are common in control populations.

GENOTYPE/PHENOTYPE CORRELATION OF XSCID MUTATIONS

The expression of functional γc is severely compromised by all but a handful of the mutations reported; exceptional cases with atypically mild CID, late presentation, or normal numbers of T cells have revealed important aspects of the molecular biology of γc. In one instance, initially normal T-cell numbers declined with time and TCR diversity was limited by an *IL2RG* substitution of an A residue for the almost invariant G nucleotide at the end of exon 1. While the missense mutation in γc encoded by this change, D39N, appeared not to impair IL-2 binding, the loss of a G terminal nucleotide in exon 1 caused most of the mRNA to be incorrectly spliced (DiSanto et al., 1994c).

In another CID kindred from Galveston, Texas, an intracellular *IL2RG* point mutation L293Q within the Box-1/Box-2 intracellular portion of γc impaired interaction with JAK3, suggesting the importance of JAK3 in the γc signaling pathway (Fig. 10.2) (Goldman et al., 2001; Russell et al., 1994; Schmalstieg et al., 1995).

Another XSCID patient was inexplicably mildly affected despite having inherited an *IL2RG* missense mutation that eliminated the fourth conserved cysteine residue (C115R), required for proper disulfide bond formation and normal configuration of all members of the cytokine receptor family (Bousso et al., 2000). Investigations revealed reversion to wild-type sequence, probably at the level of a T-lymphocyte progenitor, allowing production of functional T cells with normal γc. This case illustrated the powerful selective advantage of gene-corrected cells in XSCID, an argument in favor of retroviral gene therapy for this disease.

Two unrelated patients had XSCID with normal numbers of poorly functioning T cells and shared the missense mutation R222C; one of these had a thymus biopsy demonstrating relatively normal histology with preservation of cortical and medullary regions (Mella et al., 2000; Scharfe et al., 1997).

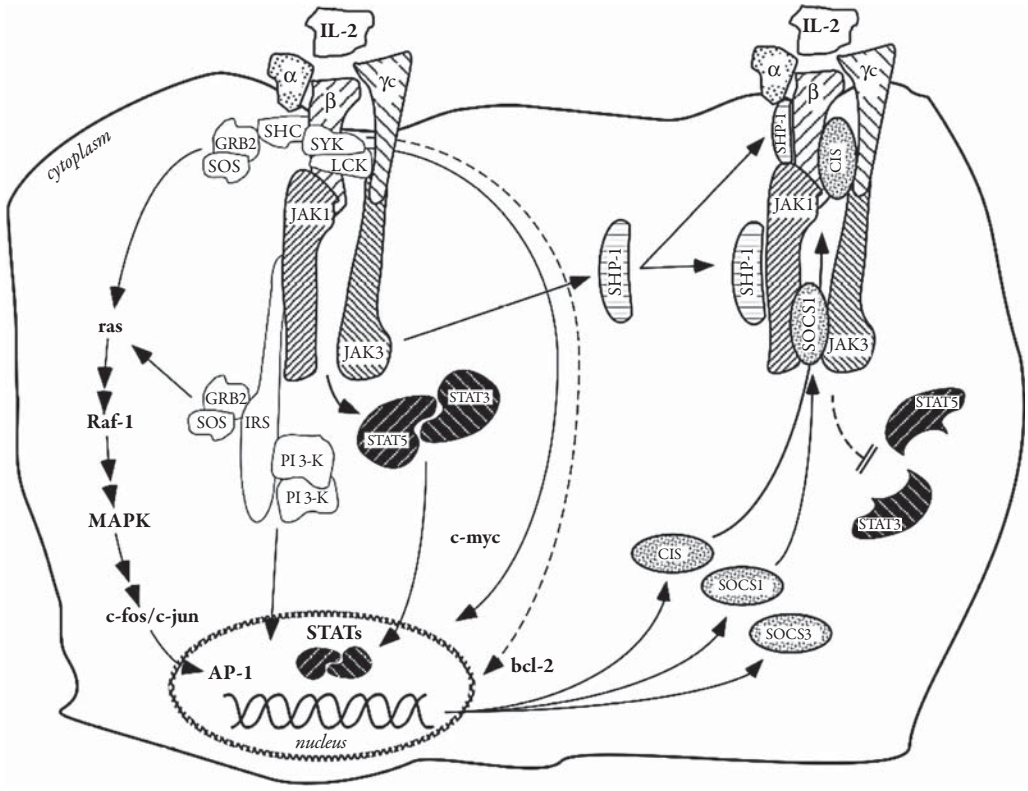

Figure 10.4 Schematic representation of the IL-2-mediated JAK-STAT signaling pathway showing proteins that participate in positive (*solid lines*) and negative (*dashed lines*) regulation.

However four other instances of this mutation have been associated with typical SCID (Clark et al., 1995; J. Puck, unpublished), as have 14 instances of the missense mutation R224W at the same amino position. Patients with CID due to missense mutation L162R (Mella et al., 2000) and a defect in the poly-A addition site of IL2RG mRNA (Hsu et al., 2000) also have been reported. In addition to having more T cells than typical XSCID patients, these individuals survived for over a decade without successful allogeneic transplantation, but resisted engraftment of allogeneic HSC, exhibited activated, DR^+ T cells and immune dysregulation, and suffered from growth delay and chronic pulmonary disease. Taken together, these natural experiments in mutagenesis indicate that low levels of normal γc or mutated γc with residual function can allow some T-cell development to occur, but also are associated with immune dysregulation.

MOLECULAR BASIS OF ADDITIONAL CYTOKINE SIGNALING DEFECTS CAUSING SCID

While the majority of patients with B^+ SCID have mutations at the *IL2RG* locus, the existence of AR B^+ SCID was indicated by the demonstration that B^+ SCID may occur also in females and in infants born to consanguineous parents (Stephan et al., 1993). Immunological and clinical features at diagnosis are similar in X-linked and AR B^+ SCID (Tables 10.1, 10.2). The finding that cytokine receptors utilizing the common γ chain always associate with the intracellular tyrosine kinase JAK3 (Fig. 10.4) and the discovery that the major cytokine receptor transducing subunit binds to another JAK kinase, JAK1 (Miyazaki et al., 1994; Russell et al., 1994), provided a clue as to the molecular bases of AR B^+ SCID. The human CID resulting from a missense mutation in the cytoplasmic tail of γc diminished its interaction with JAK3 (Russell et al., 1994). Based on this information, it was hypothesized that human AR B^+ SCID could be caused by defects of JAK3, encoded on chromosome 19p13.1 (MIM*600802). This hypothesis was proved correct by two groups who reported mutations in the *JAK3* gene in infants with B^+ SCID (Candotti et al., 1997; Macchi et al., 1995; Russell et al., 1995).

The critical role of JAK3 for lymphoid development and function has been further illustrated by the generation of Jak3-deficient mice (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995). The immunological phenotype of Jak3 knockout mice is essentially identical to that of γc knockout mice (Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996), substantiating the notion that the γc and Jak3 proteins act together.

Mice with targeted genetic disruption of *Il7r,* the gene encoding the IL-7 receptor α chain (IL7Rα), also have profound defects in T-cell development similar to γc and Jak3 knockout animals (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). This observation led to the additional hypothesis that defects in IL-7 signaling could result in SCID in humans. The search for such patients ultimately demonstrated that mutations of the human *IL7R* gene on chromosome 5p13.2

(MIM*146661) can cause AR B^+ SCID. Importantly, and in contrast to patients with γc and Jak3 deficiency, such patients retain residual development of NK cells and also have B cells that are intrinsically functional (Puel et al., 1998).

Further cases of SCID and CID were subsequently recognized in patients with mutations in intracellular mediators of cytokine signals, STAT5b, Lck (see below), and Tyk2 (see Chapter 38); additional patients with B^+ SCID have defects yet to be characterized and understood at the molecular level.

CYTOKINE SIGNALING THROUGH THE JAK-STAT PATHWAY

The effects of cytokines are mediated through interaction with specific receptors to phosphorylate specific intracellular proteins. Transmembrane receptors of the cytokine receptor superfamily do not have intrinsic kinase activity but recruit intracellular kinases following interaction with their extracellular ligands (Ihle, 1995; Leonard and O'Shea, 1998; Taniguchi, 1995). JAKs couple extracellular cytokine binding with intracellular phosphorylation of protein substrates, and thus induce cell growth and differentiation. The JAK kinases were originally cloned through a PCR-based approach (Wilks, 1989), and their functional role was first demonstrated in response to interferons (Muller et al., 1993; Watling et al., 1993). Four distinct members of the JAK family in humans are JAK1, JAK2, JAK3, and Tyk2.

The JAK family members share a C-terminal kinase domain (termed JAK homology 1, JH1), a more proximal kinase-like domain (JH2) whose function is not completely defined, and five other N-terminal regions of homology (JH3-JH7) (Fig. 10.5) (Ihle, 1995; Leonard and O'Shea, 1998; Taniguchi, 1995). The JH2 domain lacks highly conserved critical residues that are found in other typical kinase domains; when expressed in a recombinant form, JH2 is in fact devoid of kinase activity. The JAK3 cDNA is composed of 4,064 nucleotides and contains an open reading frame that encodes 1,124 amino acids with a molecular weight of 125 kDa (Kawamura et al., 1994). While JAK1, JAK2, and Tyk2 are broadly expressed, JAK3 is largely restricted to the hematopoietic system. The cDNA of JAK3 (originally designated L-JAK) was identified in NK cells and NK-like cell lines, but not in resting T lymphocytes, although expression could be induced following in vitro T-cell activation (Johnston et al., 1994; Kawamura et al., 1994). In B cells, JAK3 expression increases following in vitro activation, and it is high in B-cell malignancies (Tortolani et al., 1995). A splice variant, devoid of kinase activity, has been also identified in epithelial cells (Lai et al., 1995). Studies of murine JAK3 have revealed high levels of expression in fetal and adult thymus and lower levels in fetal liver, adult bone marrow, lymph nodes, spleen, and $CD4^+$ $CD8^+$ thymocytes (Gurniak and Berg, 1996). These data imply a crucial functional role of the JAK3 protein in lymphoid differentiation.

Intracellular signaling from all receptors of the γc superfamily (i.e., IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, IL-21R) involves physical association of γc to JAK1 and JAK3 (Foxwell et al., 1995; Johnston et al., 1995b; Malabarba et al., 1995; Miyazaki et al., 1994; Pernis et al., 1995; Russell et al., 1994; Yin et al., 1995b). The observation that distinct cytokines signal through the same set of kinases indicates that JAKs alone do not control the specificity of the signal. In the IL-2R, JAK1 interacts with the serine region of IL-2Rβ, whereas the 48 C-terminal residues of γc bind JAK3 (Miyazaki et al., 1994). Both regions of the IL-2R chains are critical for JAK activation and signal transduction (Johnston et al., 1994; Miyazaki et al., 1994; Witthuhn et al., 1994). Following cytokine/cytokine receptor interaction and dimerization of the cytoplasmic tails of the cytokine receptor chains, the JAKs are brought in close proximity and may cross-phosphorylate each other. Activation of JAK3 in response to IL-2, IL-4, and IL-7 is more pronounced than that of JAK1 (Foxwell et al., 1995; Johnston et al., 1995b; Malabarba et al., 1995; Sharfe et al., 1995; Witthuhn et al., 1994). In addition, JAKs phosphorylate the membrane distal region of the cytokine receptor chains, thus generating docking sites for SH2-containing proteins (Ihle et al., 1994; Ivashkiv, 1995; Taniguchi, 1995).

Several signaling pathways are elicited by JAK1/JAK3 activation (Fig. 10.4). First, the phosphorylated cytokine receptor may associate with the adaptor SHC (Egan et al., 1993; Karnitz and Abraham, 1995), which is itself phosphorylated and binds to Grb2, which may thus anchor to Sos, the Ras guanine nucleotide exchanging factor (Holsinger et al., 1995; Karnitz and Abraham, 1995; Li et al., 1993). Membrane translocation of the Grb2/Sos complex catalyzes the conversion of inactive, GDP-bound Ras to the active, GTP-bound state. This results in the activation of Raf-1, MAP-kinase, and eventually in induction of immediate-early genes c-fos and c-jun (Blumer and Johnson, 1994). This signaling pathway, however, is apparently not utilized by all γc receptor complexes, and in particular is not triggered by IL-4 (Welham et al., 1994).

Second, JAKs may bind and phosphorylate insulin receptor substrates (IRSs) (Johnston et al., 1995b; Keegan et al., 1995; Sharfe et al., 1995; Yin et al., 1995a). JAK activation by IL-2, IL-4, IL-7, IL-9, and IL-15 results in phosphorylation of IRS-1, whereas evidence of JAK-dependent tyrosine phosphorylation of IRS-2/4PS (which is strictly homologous to IRS-1) has so far been obtained only for IL-2, IL-4, and IL-15 (Johnston et al., 1995b; Keegan et al., 1995). The ability to phosphorylate IRS substrates is restricted to proliferating cells, as IL-4-dependent proliferative responses are not obtained with IL-4R mutants that are unable to recruit IRS-1 (Keegan et al., 1994). Once activated, IRS may bind the SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (Myers et al., 1992; Sun et al., 1995), and the catalytic activity of the p110 subunit of PI3-K is eventually elicited. In addition to promoting PI3-K activation, tyrosine phosphorylated IRS may recruit Grb2 and thus amplify the ras/Raf-1 signaling pathway (White and Kahn, 1994).

A third, essential component of the JAK signaling pathway is phosphorylation of signal transducers and activators of transcription (STAT) factors. S (Ihle, 1996; Leonard and O'Shea, 1998; Taniguchi, 1995). STATs contain a tyrosine residue that may undergo JAK-mediated phosphorylation, and they also contain SH2 and SH3 domains. Following cytokine interaction with receptor and triggering of the JAK-mediated signaling

pathway, STATs may interact with the cytokine receptor complex by binding via their SH2 domain to the phosphotyrosine of the cytokine receptor chain. In addition, following STAT phosphorylation, STAT-STAT homo- or hetero-dimerization occurs, with the SH2 domains of each STAT molecule binding to the phosphotyrosines of its partner. Six different classes of STATs have been reported; furthermore, two forms of STAT5 (STAT5a and STAT5b) have been characterized at the molecular level (Lin et al., 1996). The specificity of the response to cytokines is largely dependent on the particular combination of STATs recruited by the different signal-transducing chains of the cytokine receptor (Fenghao et al., 1995; Foxwell et al., 1995; Gilmour et al., 1995; Hou et al., 1995; Ihle, 1996; Johnston et al., 1995a; Taniguchi, 1995). However, three crucial lymphocyte growth factors, IL-2, IL-7, and IL-15, all activate STAT3 and STAT5.

Following dimerization, STATs translocate to the nucleus, where they bind to consensus sequences in the enhancer elements of the promoter regions of target genes and favor gene transcription (Ihle, 1996). Gene accessibility to STAT binding is another mechanism through which specific responses are obtained to distinct cytokines. It has been suggested that JAK-dependent STAT activation is more important to cell differentiation than to proliferation. In fact, deletion of the C-terminal H domain of IL-2Rβ abolishes IL-2-induced STAT5 activation, but not JAK1 and JAK3 activation; nor does it affect cell proliferation (Fujii et al., 1995). However, IL-4-induced thymocyte proliferation is somewhat diminished in STAT6 knockout mice, suggesting that STAT activation contributes to cytokine-induced growth-promoting activity (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996).

Additional, possibly JAK-independent, signaling pathways that are elicited following cytokine/cytokine receptor interaction include activation of src-related kinases (Lck, Lyn, and Fyn in response to IL-2) (Taniguchi and Minami, 1993), through their association with the acidic domain of IL-2Rβ. Deletion of this domain abolishes IL-2-induced Lck activation yet does not affect IL-2-regulated cell growth, implying that activation of src-related kinases may not be crucial for cell proliferation. Finally, induction of Syk, c-myc, and Bcl-2 requires the S region of IL-2Rβ (Minami and Taniguchi, 1995). JAK3 mutants devoid of the JH1 kinase domain cause markedly diminished induction of c-myc but intact induction of Bcl-2, indicating that these are independent signaling pathways and that JAK3 may be involved in c-myc transcription (Kawahara et al., 1995).

The need for JAK3 in IL-2-induced cell proliferation has been illustrated by the observation that in the NIH3T3αβγ cell line (which expresses the α and β chains of the IL-2 receptor and γc, as well as JAK1 and JAK2, but not JAK3), IL-2 does not promote proliferation; however, proliferative response to IL-2 is restored after JAK3 is transfected and expressed by these cells (Miyazaki et al., 1994). Furthermore, the requirement for integrity of the structure of JAKs in regulating cell growth and differentiation has been indicated by the fact that JAK3 mutants that lack the JH3-JH7 domains (Fig. 10.5), but not the JH1 protein tyrosine kinase domain, are unable to interact with γc (Taniguchi, 1995), whereas mutants that lack the JH1 kinase domain may bind to γc but are unable to transduce activation signals (cell growth, induction of c-fos and c-myc) in response to IL-2 (Taniguchi, 1995). Finally, overexpression of JAK3 mutants that lack the JH1 kinase domain may inhibit the IL-2-induced phosphorylation of JAK1 and JAK3 (Kawahara et al., 1995). Similar experiments with JAK2 mutants suggest that multiple JH domains are required for functional interaction with the cytokine receptor and target cytoplasmic substrates (Tanner et al., 1995).

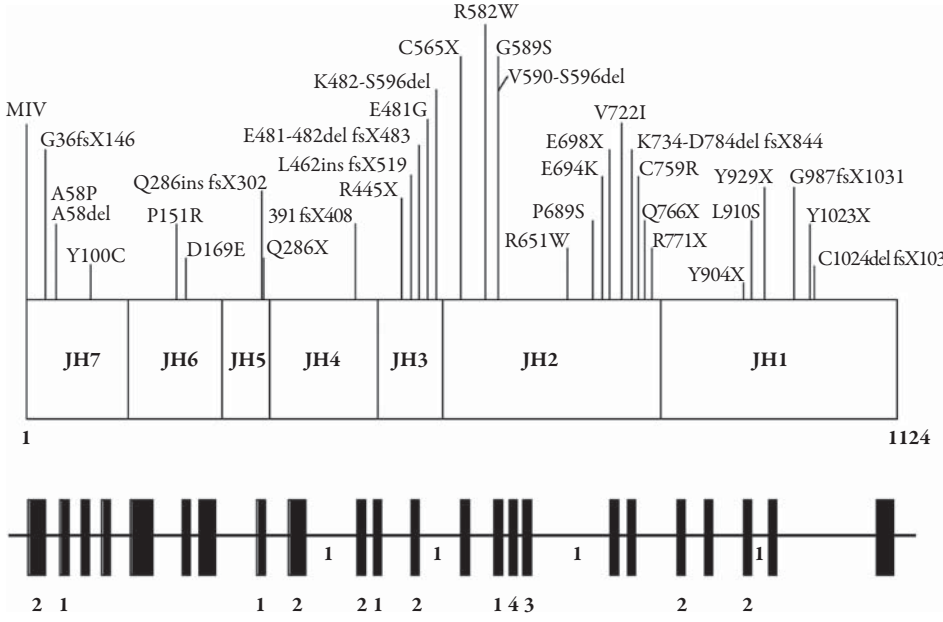

Figure 10.5 Representation of the human JAK3 protein (above), with boxes representing the JAK homology domains (JH1–JH7), observed patient mutations, and *JAK3* gene organization (below), with exons (*vertical rectangles*), introns, and numbers of *JAK3* mutations observed in each as reported through 2006.

A number of mechanisms serve to terminate the activity initiated by cytokine signals (Fig. 10.4). SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 are important inhibitors of signaling events and are thought to dephosphorylate receptor chains and JAK molecules through SH2–phosphotyrosine interaction (Migone et al., 1998; You et al., 1999). Other mechanisms for attenuation of cytokine signaling involve the members of the suppressor of cytokine signaling (SOCS) family of proteins. These proteins are induced by cytokine stimulation following a feedback mechanism. Eight members of this family are known (CIS and SOCS1-7) (Endo et al., 1997; Hilton et al., 1998; Naka et al., 1997; Starr et al., 1997; Yoshimura et al., 1995). SOCS proteins appear to inhibit cytokine signaling by a variety of mechanisms, including competition with STATs for the phosphorylated docking sites on receptor chains and inhibition of kinase activities.

CLINICAL FEATURES AND LABORATORY FINDINGS OF SCID DUE TO MUTATIONS OF JAK3

By 2004, many publications described SCID patients with JAK3 defects (Bozzi et al., 1998; Buckley et al., 1999; Candotti et al., 1997; Frucht et al., 2001; Macchi et al., 1995; Mella et al., 2001; Roberts et al., 2004; Russell et al., 1995; Schumacher et al., 2000). Their clinical features were indistinguishable from those of infants with X-linked SCID (Tables 10.1 to 10.3). In two cases, the diagnosis of SCID was established in healthy infants immediately after birth, either because of family history of SCID or because of fortuitous recognition of reduced circulating T cells. Most patients, however, were diagnosed following development of upper and lower respiratory tract infections, chronic diarrhea, central nervous system involvement, and failure to thrive during the first few months of life. In all cases, peripheral lymph nodes were undetectable. Although splice variants of JAK3 mRNA have been identified in nonhematopoietic human tissues (Lai et al., 1995), no unique manifestations of primary organ dysfunction unrelated to immune deficiency have been detected in JAK3-deficient infants. Parental consanguinity was documented in several cases.

The clinical and pathological features as well as laboratory findings of JAK3-deficient patients are very consistent with X-linked SCID (Tables 10.1 to 10.3). In addition to being severely reduced in number, T cells from JAK3-deficient infants are functionally impaired. The proliferative responses to PHA and other mitogens, antigens, and alloantigens are abolished or severely reduced. Despite the increased proportion of circulating B cells, serum immunoglobulin levels are low (with the exception of IgG that is maternally derived during the early months of life), and no antibody responses are elicited following antigen stimulation. Failure to generate antibody responses is partly due to lack of effective helper T-cell activity, but it also reflects an intrinsic B-cell defect. In fact, defective induction of STAT activation has been reported following cytokine stimulation in Epstein-Barr virus (EBV)-transformed B cells from these patients (Candotti et al., 1996; Frucht et al., 2001; Oakes et al., 1996; Russell et al., 1995).

As with XSCID, the immunological phenotype, lack of circulating T cells, and increased proportion of B cells may change during the course of JAK3-deficient SCID. One infant who had a typical $T^- B^+ NK^-$ phenotype during the first month of life had a rapid appearance of T cells, up to 41 percent of total circulating lymphocytes, that were oligoclonal, $CD4^+$ $CD45R0$ DR^+ cells that failed to respond to PHA or CD3 stimulation alone, but showed some response to the combination of anti-CD3 and IL-2 (Brugnoni et al., 1998). At the same time that these T cells were found, the patient developed elevated IgE (1,000 IU/mL, with normal levels being <100 IU/mL). This phenotype of partial preservation of the responsiveness to IL-2 by activated T cells correlated with *JAK3* gene mutations (A1537G and deletion of exons 10–12) that permitted residual protein expression and function (Candotti et al., 1997). On the other hand, the contributory role of residual functional activity of JAK3 is less clear if one considers that Jak3 knockout mice develop activated CD4 T cells (see below). It is therefore possible that the T-cell differentiation defect imposed by human JAK3 deficiency allows changes of the immunological phenotype with time or that some degree of T-cell development is possible independently from JAK3.

It is also recognized that JAK3 is expressed in myelomonocytic cells, including human monocytes (Musso et al., 1995; Witthuhn et al., 1994); in vitro stimulation with lipopolysaccharide (LPS) and interferon increases JAK3 expression in monocytes (Musso et al., 1995). Furthermore, IL-2 and IL-4, two cytokines that signal through γc-bearing receptors, have profound effects on cells of the monocyte–macrophage lineage (de Waal Malefyt et al., 1993; Fenton et al., 1992; Vannier et al., 1992) and induce tyrosine phosphorylation and activation of JAK3 in these cells (Musso et al., 1995; Witthuhn et al., 1994). The possibility that mutations of the *JAK3* gene might affect the differentiation and/or function of the myelomonocytic lineage has been considered, but monocyte numbers are normal in JAK3-deficient SCID infants and showed normal responses to IL-2 (measured as release of TNF and IL-8) and to IL-4 (release of IL-1R antagonist) (Villa et al., 1996), indicating that JAK3 is dispensable for monocyte differentiation and responsiveness to cytokines that interact with γc-user receptors.

MOLECULAR BASIS OF JAK3 DEFICIENCY

As with XSCID, there is great diversity in *JAK3* gene mutations, which include missense, nonsense, and splice site mutations as well as less common genomic deletions and insertions. Most *JAK3* mutations drastically affect the expression of the protein; however, a number of missense or small in-frame deletions have been identified that permit near-normal levels of expression of the protein (Notarangelo et al., 2001; Roberts et al., 2004). The analysis of the effect of these mutations has provided important insights on the function of the different JAK3 domains (Fig. 10.5).

Not unexpectedly, mutations of the kinase domain (JH1) can affect phosphorylation of JAK3 and its substrates IL2Rβ

and STAT5, as was demonstrated in patients with nonsense and frameshift mutations (Notarangelo and Candotti, 2000; Schumacher et al., 2000). More interesting have been the consequences of the frequent mutations in the pseudokinase domain (JH2). Constitutive phosphorylation of JAK3 was detectable in a patient with a C759R missense mutation in the JH2 domain, yet IL-2 stimulation did not result in upregulation of JAK3 phosphorylation, nor did it induce STAT5 phosphorylation.

The functional effects of mutations have also been evaluated with heterologous systems. Mutant JAK3s were normally expressed after cDNA transfection, but their kinase activity was undetectable in vitro (Chen et al., 2000). Moreover, JAK3 mutants were unresponsive to IL-2 stimulation, even though they could normally bind to γc. Surprisingly, however, the mutated JAK3 appeared in some cases to be hyper-tyrosine phosphorylated compared with wild-type JAK3, and suggesting that the physiological role of the JH2 pseudokinase domain is to regulate kinase activity and therefore substrate phosphorylation by directly interacting with the JH1 kinase domain. To confirm this relationship, it was shown that, whereas the wild-type JAK3 pseudokinase domain modestly inhibited the JAK3-mediated signaling pathway, JAK3 with the mutated JH2 pseudokinase domains from two patients had an increased capacity to inhibit kinase activity. Thus, kinase dysregulation in these patients appeared likely to have contributed to their disease pathogenesis (Chen et al., 2000).

Mutations in the JH3 domain are also compatible with residual JAK3 expression, as in the case of a patient who carried one missense mutation (E481G) and one deletion (K482-S596del) in this domain predicted to result in both a normal-sized and a low-molecular-weight mutant JAK3 product. In EBV-B cells from this patient, IL-2 induced some residual phosphorylation of the normal-sized mutant JAK3 product. Furthermore, STAT5 phosphorylation was also detected (although at reduced levels), indicating that the glutamic acid-to-glycine substitution at codon 481 in the JH3 domain did not completely abrogate JAK3 function. Interestingly, the immunological phenotype of this patient was atypical, as he developed a substantial number of autologous T cells (although with abnormal phenotype and function) and NK lymphocytes (Brugnoni et al., 1998; Candotti et al., 1997).

Mutations in the N-terminal of JAK3 have indicated the important role that this portion of the protein plays for receptor interaction. Cells from patients with single amino acid substitution or deletion in the JH7 domain express JAK3 mutants whose interaction with γc is severely affected (Cacalano et al., 1999) and with abrogated in vitro catalytic activity. Similar results were observed in the presence of missense mutations affecting the JH2 domain (Roberts et al., 2004). A detailed molecular analysis utilizing constructs carrying the SCID-associated FERM mutation has shown that the JAK3 FERM and kinase domains associate and reciprocally influence each other's function and structure. Thus, in SCID patients with FERM mutations, two mechanisms contribute to the disease pathogenesis: impaired γc/JAK3 association and inactivation of catalytic activity.

SCID DUE TO MUTATIONS OF IL-7 RECEPTOR α CHAIN (IL7Rα)

Patients with IL7Rα deficiency present with classical signs and symptoms of SCID as listed in Tables 10.1 and 10.2. The only difference in laboratory abnormalities is that patients with IL7Rα defects generally have normal numbers of functional NK cells (Table 10.3). The original report (Puel et al., 1998) described two patients with $T^- B^+ NK^+$ SCID characterized by failure to thrive, recurrent otitis, viral infections, candidiasis, diarrhea, and fever. A subsequent report described three affected subjects in an additional consanguineous family; the infants had thrush, absence of palpable lymph nodes, and no thymus shadow on a chest radiograph (Roifman et al., 2000). In a series of 169 SCID cases diagnosed at Duke University Medical Center, 16 (9.5 percent) had IL7Rα-deficiency, making this the third most common cause of SCID in that series after X-SCID and ADA deficiency (Buckley, 2004).

MOLECULAR BASIS OF IL7RA DEFICIENCY

IL7Rα (CD127) is, like γc, a type 1 cytokine receptor chain. This 460 amino acid protein can bind IL-7 with consequent dimerization with γc, which in turn results in JAK3-mediated phosphorylation of IL7Rα and recruitment of JAK2 and STAT5. The *IL7R* gene, like *IL2RG*, is organized into 8 exons and 7 introns, with exons 1 through 5 encoding the extracellular portion of the receptor, and exon 6 and exons 7 and 8 encoding the transmembrane region and the intracellular tail, respectively. The cDNA is 1,380 nucleotides long and encodes a protein of the estimated molecular weight of ~90 kDa. The expression of *IL7R* mRNA can be detected in the earliest stages of lymphocyte differentiation ($CD4^- CD8^-$ double-negative thymocytes and $c\text{-}kit^+ B220^+$ B-cell progenitors) (Orlic et al., 1997; Sudo et al., 1993).

Mutations of the *IL7R* gene are heterogeneous and include missense, nonsense, and splice mutations as well as an intron mutation that introduced a cryptic splice site (Butte et al., 2007).

MURINE MODELS OF IL7Rα DEFICIENCY

IL7Rα participates in both the IL-7 and thymic stromal lymphopoietin (TSLP) cellular receptors, in association, respectively, with γc and TSLPR, a receptor chain with 24 percent homology to γc (Pandey et al., 2000; Park et al., 2000). IL7Rα knockout mice, therefore, lack both IL-7 and TSLP signaling. These mice are viable and fertile, and retain normal proportions of all major thymocyte populations during fetal life (Crompton et al., 1998). Adult IL7Rα-deficient mice, however, have ~20-fold reduction of thymic precursors and decreased peripheral lymphocyte numbers (Peschon et al., 1994). Thymocyte development is arrested at the $CD4^- CD8^-$ double-negative stage, before the occurrence of TCR gene rearrangement (Candeias et al., 1997; Crompton et al., 1997;

Maki et al., 1996a; Perumal et al., 1997). The reduced numbers of T cells that populate the periphery are characterized by impaired survival and reduced proliferation to mitogens and alloantigens (Maraskovsky et al., 1996). Development of γδ T cells is also blocked in IL-7Rα-deficient mice, while NK cells develop normally (He and Malek, 1996; Kang et al., 1999; Maki et al., 1996b). Interestingly, thymocyte development can be restored by the introduction of a transgenic TcR, suggesting that IL-7Rα is needed to initiate TcR gene rearrangement (Crompton et al., 1997). On the other hand, T lymphopoiesis is also restored in IL-7Rα-deficient mice by the transgenic expression of the anti-apoptotic molecule bcl-2 and ameliorated by the concomitant absence of the Bax pro-apoptotic protein, indicating that the critical consequence of engagement of IL-7Rα in T-cell development may be the integration of cell-survival signals (Akashi et al., 1997; Khaled et al., 2002; Maraskovsky et al., 1997).

IL7Rα-deficient mice also show an incomplete block of B-cell development at the transition from the pro-B to pre-B cell stage, which results in a 10-fold reduction in precursor B cells and severely decreased numbers of mature, peripheral B lymphocytes expressing surface Ig (Peschon et al., 1994). This defect is not corrected by transgenic expression of Bcl-2 (Maraskovsky et al., 1998) and is characterized by normal D-J segment joining but impaired recombination of antibody heavy-chain V segments (Corcoran et al., 1998). The reduction of B-lymphocyte numbers in IL7Rα-deficient mice does not parallel the human phenotype. This contrast may be attributable to the fact that the essential role of TSLP in maturation of pre-B cells in mice (Friend et al., 1994; Levin et al., 1999) is not conserved in humans (Reche et al., 2001; Soumelis et al., 2002).

STRATEGIES FOR DIAGNOSIS OF SCID OR CID

Once the clinical suspicion of a combined T- and B-cell immunodeficiency has been raised, whether by population-based screening, family history, low lymphocyte count, or recurrent or opportunistic infections, the diagnosis of SCID or CID must be made by lymphocyte phenotyping and functional tests, with specific mutation diagnosis highly desirable. Clues as to the specific *IL2RG*, *JAK3*, or *IL7R* genotype can be gleaned from the immunological tests, but atypical profiles of lymphocyte subsets can occur. Males with very few T cells, high proportions of B cells, and absent NK cells are likely to have *IL2RG* defects, although AR *JAK3* mutations have an identical clinical and immunological picture.

Lymphocyte phenotyping can be misleading in the face of maternal T-cell engraftment, transfusion of nonirradiated blood products, and/or infectious agents. Deficiency of naïve versus memory phenotype of CD3 T cells, assessed by using the cell-surface markers such as CD45RA and CD45RO, respectively, is characteristic of SCID or CID. Additional testing of the isolated T-cell population with DNA markers can distinguish autologous from allogeneic cells.

The availability of an anti-γc monoclonal antibody makes it possible to evaluate patient peripheral blood lymphocytes for γc expression (Puck et al., 1997a; Gilmour et al., 2001a). If γc is clearly absent from patient cells as compared to controls, XSCID can be suspected. However, the presence of γc may indicate that the patient's *IL2RG* gene is encoding an expressed but nonfunctional protein, or that maternal or other foreign cells in the infant's peripheral blood are positive for anti-γc staining.

Molecular testing for XSCID in the past used indirect methods, linkage and maternal lymphocyte X chromosome inactivation, and even after identification of *IL2RG* as the XSCID disease gene, mutant exons were identified by differential annealing of wild-type and variant strains. At this time, however, indirect methods are complex, expensive, time-consuming and not always interpretable or definitive. Although critical for the gene hunt that identified *IL2RG*, they are now appropriately replaced by DNA sequence-based analysis (Puck et al., 1997b). Genomic sequence analysis of PCR-amplified DNA reveals the specific mutation in patients and carrier relatives. If a mutation has been previously published with functional data, no further workup is needed, while new variants in sequence require validation as disease-causing mutations.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

Females who carry an *IL2RG* mutation on one of their X chromosomes are immunologically indistinguishable from controls. However, they can be identified by nonrandom X chromosome inactivation, which is seen in their lymphocytes but not in their granulocytes or nonlymphoid cells (Conley et al., 1988). This skewed X inactivation is a result of the selective disadvantage of lymphocyte precursors that have inactivated the X chromosome with an intact *IL2RG* gene early in embryogenesis. While X inactivation was formerly used as a carrier test, direct detection of mutations by sequencing for all SCID genes is now preferred. Females carrying a pathogenic *IL2RG* mutation have a 50 percent risk of XSCID for each male pregnancy and a 50 percent risk that each female pregnancy will also carry the mutation. However, as expected with X-linked lethal disorders, new mutations are common and failure to find a mutation in maternal blood does not preclude a mutation in the germline that may be inherited by multiple offspring. Such female germline mosaicism has been documented in XSCID (O'Marcaigh et al., 1997). In 13 percent of 85 mothers of sons with proven XSCID mutations, the mutations were not present in maternal blood samples (Puck et al., 1997a). Because the mutations in such cases may have existed in only a single maternal oocyte or may represent extensive maternal mosaicism, the recurrence risk for these mothers must be given as between 0 and 50 percent for a subsequent male pregnancy. New mutations have also been documented to occur in AR SCID.

Prenatal diagnosis can be performed by several methods, depending on the amount of information available about the genotype of the family requesting it. If specific mutation information is known, sequencing can be performed using chorionic villus sample (CVS) or amniocyte DNA prepared directly or

obtained from cultured cells (Puck et al., 1990, 1997b). When the genotype of a deceased SCID proband is not known, fetal blood sampling has also been used. Although this procedure must be done later in pregnancy and entails more risk than CVS or amniocentesis, lymphocytopenia, low numbers of T cells, and poor T-cell blastogenic responses to mitogens can be definitively demonstrated in affected fetuses by week 17 of gestation (Durandy et al., 1986).

No carrier or prenatal testing should take place without genetic counseling. In considering prenatal diagnosis, the diagnostic options should be weighed against testing at birth for families who would not terminate an affected pregnancy. Regardless of whether prenatal testing is undertaken, education and counseling can clarify the potential benefit of early HCT for affected infants. Relatives of SCID patients who did not survive in the past may not be aware of the medical advances in transplantation therapy.

Puck et al. (1997b) studied the utilization of prenatal diagnosis for XSCID. The great majority of families at risk for having an affected pregnancy desired prenatal testing, whether or not termination of pregnancy was a consideration. In fact, in only 2 instances out of 13 predicted affected male fetuses did families terminate a pregnancy. To prepare for optimal treatment of an affected newborn, families and their medical providers selected bone marrow transplant centers, undertook HLA testing of family members, and even began a search for a matched, unrelated bone marrow donor.

TREATMENT AND PROGNOSIS

SCID is generally fatal unless the immune system can be reconstituted, either by allogeneic transplantation or correction of autologous hematopoietic cells by gene therapy. Infants receiving allogeneic HCT soon after birth are less likely to have serious pretransplant infections or failure to thrive. They also appear to have more rapid engraftment, fewer posttransplant infections, less GVHD, and shorter hospitalizations than those whose transplants are delayed (Antoine et al., 2003; Buckley, 2004; Giri et al., 1994b; Meyers et al., 2002). Supportive care and management of infections from the time of diagnosis through the transplantation period are essential. Intensive monitoring, parenteral nutrition, immunoglobulin replacement, and antibiotics, including antifungal and antiviral agents, have dramatically improved the survival of infants with SCID. Patients should be isolated from exposure to infectious agents in the environment and should not receive live vaccinations. Attempts to maintain a totally aseptic "bubble" environment are not indicated; instead, prompt immune reconstitution should be the goal.

The best current treatment for SCID is HCT from an HLA-matched related donor, but unfortunately most patients lack such a donor. Haploidentical, T-cell-depleted HCT has proven successful (see Chapter 60). Transplantation protocols using matched unrelated adult bone marrow or cord blood stem cells are also successful. The techniques for eliminating mature T cells from the donor-cell population and enriching for stem cells vary between centers. Similarly, different centers have had different approaches regarding pretransplant chemotherapy or posttreatment GVHD prophylaxis for SCID patients. The potential advantages of controlling GVHD from donor immunocompetent cells and clearing out host bone marrow in the hope of improving donor stem-cell engraftment and B-cell production must be weighed against the risks of cytotoxic treatment.

Previously there were few useful data to compare the regimens used, but now increased numbers of treated patients and specific mutation diagnosis for most infants with SCID allow these questions to be addressed. Nevertheless, it is not yet possible to predict which patient may develop GVHD, fail to make adequate antibodies and require long-term immunoglobulin replacement, or eventually develop T-cell dysregulation or decreased T-cell function. Some of the oldest surviving individuals with SCID received HLA-matched related bone marrow transplants and are now in their forties and in excellent health. As larger numbers of children are now growing up after transplantation for SCID, long-term outcome data are becoming available (Antoine et al., 2003; Buckley, 2004, 2011). While a large proportion of subjects remain healthy, some continue to require immunoglobulin replacement, and some eventually experience declines in thymic output, decreased naïve T-cell numbers, and diminished TCR diversity (Antoine et al., 2003; Buckley, 2011; Buckley et al., 2004; Railey et al., 2012). Multicenter collaborative efforts, including that of the Primary Immune Disease Treatment Consortium in the United States, are engaged in prospective, retrospective, and cross-sectional research to probe which treatments are most successful for particular patients with SCID and CID (Griffith et al., 2008).

The concept of prenatal treatment for XSCID has been controversial due to the risk of invasive procedures during pregnancy. Advantages of in utero treatment include early reconstitution, a protected intrauterine environment, and the possibility of introducing normal bone marrow stem cells at the gestational age when fetal hematopoiesis is shifting from fetal liver to bone marrow. Early attempts at human in utero bone marrow transplantation were compromised by technological limitations, septic complications, and GVHD. In at least two patients with XSCID, these difficulties have been overcome (Flake et al., 1996). Fetuses shown by molecular diagnosis to be affected with XSCID were infused intraperitoneally with haploidentical, T-cell-depleted, $CD34^+$ positively selected paternal bone marrow cells between 17 and 20 weeks of gestation. In each of these two cases, infants were born with engrafted, functional T cells from their donors, whereas the XSCID mutation could still be confirmed in the infants' granulocytes. In long-term follow-up both children were reported to have fully reconstituted immunity without requiring immunoglobulin supplementation (Bartolomé et al., 2002; Beggs et al., 2003).

NEW THERAPIES FOR SCID

XSCID and ADA SCID have been successfully treated by gene transfer therapy to hematopoietic stem cells, but serious

adverse events have also occurred (Haeren-Bey-Abina et el., 2002). Advantages of SCID as a pilot disease for gene therapy as well as complications that have come to light since initial positive results are discussed in detail in Chapter 61.

The first clinical trials of human XSCID gene therapy at the Necker Hospital in Paris by Cavazzana-Calvo et al. (2000) enrolled infants with no available HLA-matched donor. Bone marrow from the affected patients was aspirated, enriched for stem cells by positive selection with the cell-surface marker CD34, cultured in activating cytokines, exposed to a retrovirus encoding a correct copy of *IL2RG* cDNA, and reinfused into the patients, who received no myelosuppressive treatment (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002; Fischer et al., 2011). Although several of the first patients treated became fully reconstituted with T cells and some also developed the ability to make antibody responses, an unanticipated adverse event, leukemic proliferations of T-cell clones bearing *IL2RG* retroviral insertions near a T-cell oncogene, developed. Retroviral vectors have been redesigned to improve safety, and new clinical trials have been undertaken.

Another promising technology is gene correction in autologous patient HSCs. Zinc finger nucleases, and more recently TALEN nucleases, have been designed to excise mutant DNA from patient HSCs, replacing it with the correct gene sequence (reviewed in Porteus, 2011). This approach is still in preclinical trials to optimize efficacy and safety but may offer potential for correction of any SCID genotype.

IL-2R/IL-15Rβ DEFICIENCY

IL-2 and IL-15 signal through cellular receptors that share both γc (CD132) and IL2Rβ/IL5Rβ chain (CD122). Their receptors are distinguished by their ligand-specific partners, IL2Rα (CD25) and IL15Rα, respectively. IL-2 is predominantly produced by activated T cells and plays an essential role in control of peripheral self-tolerance (Nelson, 2002) through activation-induced cell death (Frucht et al., 2001), thymic selection (Bassiri and Carding, 2001) and maintenance of regulatory T cells (McHugh et al., 2001; Shevach, 2000; Shevach et al., 2001). After binding to its receptor, IL-2 activates JAK1, JAK3, and STAT5.

Despite its shared receptor usage with IL-2, IL-15 has a quite distinct function, maintenance of $CD8^+$ memory T cells that gradually decline after viral infections in the absence of IL-15 (Becker et al., 2002; Goldrath et al., 2002; Schluns et al., 2002). IL-15 is also important for the development of NK cells, as illustrated by the lack of NK cells in IL-15 knockout mice and IL-15Rα knockout mice (Becker et al., 2002; Goldrath et al., 2002; Mohamadzadeh et al., 2001). Thus, the NK-cell deficiency observed in XSCID and JAK3-deficient patients is thought to be due to lack of IL-15 signaling.

One patient with T^{low} B^+ NK^- CID presented with undetectable expression of the β chain of the cellular receptor used by both IL-2 and IL-15 (Gilmour et al., 2001b). The infant had severe infections, including respiratory syncytial virus bronchiolitis, *Candida* enteritis, and meningoencephalitis. He had failure to thrive, hepatomegaly, and moderate lymphopenia. Immunophenotype analysis showed markedly reduced T-cell numbers and absent NK cells, with B lymphocytes within normal range. Serum immunoglobulin levels were normal, although the patient failed to produce specific antibodies following immunization with tetanus toxoid and *Haemophilus influenzae* vaccines. The patient underwent allogeneic bone marrow transplantation with good immune reconstitution and resolution of clinical symptoms.

Flow cytometry and Western blot analyses showed a significant decrease of IL-2Rβ/IL-15Rβ expression in the patient's PBMCs. No abnormalities of the *IL15R* sequence were demonstrated, and whether this clinical presentation was due to undetected mutations of the IL-2Rβ/IL-5Rβ chain *IL15R* itself or a secondary defect remains unclear. It is interesting to note that mice lacking IL-2Rβ/IL-5Rβ have defective NK-cell development, as observed in this patient. In contrast, T cells of IL-2Rβ/IL-5Rβ-deficient mice are abnormally activated, resulting in dysregulated differentiation of B cells into plasma cells and consequent high levels of serum immunoglobulins, as well as autoantibodies that cause hemolytic anemia (Suzuki et al., 1995).

LCK DEFICIENCY

Lck (p56lck) is an intracellular protein tyrosine kinase that is critically involved in TCR-mediated signaling. Its N-terminal region associates with the cytoplasmic tail of the CD4 and CD8 co-receptors (Chow and Veillette, 1995), thus bringing Lck into close proximity with the TCR. Lck is known to contribute to tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR complex (Iwashima et al., 1994; van Oers et al., 1996). In keeping with the role played by Lck in early steps of TCR-mediated signaling, targeting of the Lck gene (Molina et al., 1992), or expression of a dominant negative Lck transgene (Levin et al., 1993), results in a severe T-cell developmental defect, with profound thymic atrophy, a dramatic reduction in the number of double-positive thymocytes, and only few circulating mature T cells.

One infant with a putative Lck defect has been reported to date (Goldman et al., 1998). The patient presented with chronic diarrhea and failure to thrive at 1 month of age and developed *Enterobacter cloacae* sepsis, cytomegalovirus infection, and persistent oral candidiasis. Laboratory investigation disclosed panhypogammaglobulinemia, lymphopenia with a low proportion (9–22 percent) of $CD4^+$ T cells, and a progressive decline in T-cell proliferation to mitogens and anti-CD3 monoclonal antibody. $CD8^+$ T cells, if activated with anti-CD3, failed to express the activation marker CD69, whereas activation with phorbol esthers and ionomycin resulted in normal CD69 expression, indicating a TCR signaling defect proximal to protein kinase C activation. A marked reduction in the level of Lck and an improperly spliced Lck transcript, lacking the exon 7 coding domain, were demonstrated. The milder immunological phenotype observed in the patient when compared with gene-targeted lck knockout mice could be explained by residual, although low, expression of Lck or species differences in redundancy

of related signaling kinases. The clinical course of the infant was typical for SCID; he ultimately required bone marrow transplantation, which was reported to result in a complete cure.

SHORT STATURE AND IMMUNODEFICIENCY DUE TO MUTATIONS OF STAT5b

Six patients in five families, most of them consanguineous, have been reported who had STAT5b deficiency resulting in postnatal growth failure, facial dysmorphism, and immunodeficiency that can be characterized as CID (Bernasconi et al., 2006; Hwa et al., 2005, 2007; Kofoed et al., 2003; Rosenfeld et al., 2004; Vidarsdottir et al., 2006). The original reported case, a daughter of consanguineous healthy parents of normal stature, had severe growth failure with growth hormone insensitivity. By the age of 16.5 years she was the height of an average 6.5-year-old. She also suffered from chronic diarrhea, generalized eczema, and respiratory ailments, periodically requiring oxygen supplementation. A lung biopsy indicated the diagnosis of lymphoid interstitial pneumonia, and the patient was treated with oral steroids. At age 8 she developed severe, hemorrhagic varicella, followed by multiple episodes of herpes zoster. At age 10, progressive worsening of her pulmonary function occasioned a repeat biopsy, in which *Pneumocystis carinii* and *Rodococcus equis* were isolated. Institution of appropriate treatment and chronic management of combined immunodeficiency have resulted in an improved quality of life.

The additional patients all had significant infections and short stature with normal growth hormone, reflecting the critical role of STAT5b in signaling from the growth hormone receptor, which along with γc and IL7Rα is a member of the cytokine receptor family. Other associated features included ichthyosis and juvenile arthritis, each in one patient, and abnormal platelet aggregation in another.

LABORATORY FINDINGS AND MOLECULAR BASIS OF STAT5b DEFICIENCY

High levels of endogenous growth hormone, low insulin-like growth factor, normal growth hormone binding protein, and normal growth hormone receptor (GHR) coding sequence pointed to an intracellular defect distal to the GHR, a member of the cytokine receptor gene superfamily that acts through phosphorylation of STAT5b. Indeed, expression of STAT5b was only poorly detectable in patient fibroblasts, which failed to show phosphorylation of the protein in response to GH. STAT5a expression and ability to be phosphorylated were normal. Details about the patient's lymphocyte profile and immune functional studies have not been published.

This patient was found to be homozygous for the missense mutation in the *STAT5B* gene on human chromosome 17q11.2. The mutation is due to the substitution of C for the G nucleotide at cDNA 2057 in the alanine 630 codon, changing alanine to proline (A630P). The mutation lies in the highly conserved SH2 domain of STAT5b. Although protein is produced (but poorly recognized by some monoclonal antibodies), it is unable to be phosphorylated, preventing dimerization, translation to the nucleus, and activation of transcription.

The coexistence of extreme short stature and combined immunodeficiency may suggest STAT5b deficiency, particularly in a consanguineous family. Because of its extreme rerity, the clinical spectrum of severity of the disease is not known. The multiple roles of STAT5b in many tissues indicate that bone marrow transplantation would not be expected to correct the entire phenotype.

FUNCTIONAL ASPECTS AND ANIMAL MODEL OF STAT5B DEFICIENCY

STAT5a and STAT5b are transcription factors that are 96 percent homologous to each other and are tandemly located in both human and mouse, suggesting that these genes arose by gene duplication. The precise role of each of the two *STAT5* genes is not completely worked out in human or mouse. Mouse knockouts of STAT5a show impaired prolactin-dependent mammary gland development (Liu et al., 1997), while STAT5b knockout mice have a phenotype similar to that observed in GHR-deficient mice (Udy et al., 1997). Deletion of both STAT5a and STAT5b results in a phenotype closely resembling that observed in prolactin receptor-deficient mice, including decreased body size in males, anemia, and impaired mammary gland development, reflecting the roles of STAT5 proteins as intracellular transducers of signals from growth hormone, erythropoietin, and prolactin receptors. In addition, STAT5a and STAT5b double knockout mice show a profound T-cell proliferation defect, similar to that observed in IL2RB-deficient mice (Teglund et al., 1998). Partial T-cell defects can be observed in both STAT5a and STAT5b single knockout mice (Imada et al., 1998; Nakajima et al., 1997), indicating that the two STAT5 proteins play critical, redundant roles in peripheral T-cell activation and proliferation.

IL2RA DEFICIENCY

IL2Rα was the first chain of the IL2R complex to be cloned (Cosman et al., 1984; Leonard et al., 1984; Nikaido et al., 1984). It is a 55-kDa protein with an extracellular portion containing 219 amino acid residues, a transmembrane segment of 19 residues, and a short cytoplasmic portion containing only 13 residues. IL2Rα, unlike the other chains of this receptor, has no similarity to the cytokine receptor superfamily, but it appears to have structural homology to the α chain of the IL-15 receptor (Giri et al., 1995). Of the three chains of the IL2R, IL2Rα shows the most tightly regulated expression. During T-cell development IL2Rα is already expressed on the earliest triple-negative ($CD3^-$ $CD4^-$ $CD8^-$) thymocytes (Godfrey et al., 1993), but subsequently it is dramatically downregulated. In resting, mature T cells IL2Rα is not expressed; it is induced

by stimulation through the TCR or by IL-1 and TNFα (Crabtree, 1989; Rothenberg, 1991). Similarly, mature B cells newly express IL2Rα following stimulation through the B-cell receptor (Jung et al., 1984; Tsudo et al., 1984; Waldmann et al., 1984). IL2Rα is also upregulated in response to IL-2.

CLINICAL AND IMMUNOLOGICAL PHENOTYPE OF IL2RA DEFICIENCY

To date only two human subjects from a single consanguineous family have been identified with IL2Rα deficiency (Roifman, 2000; Sharfe et al., 1997; unpublished observations). The index patient, a male, presented with persistent thrush, Candida esophagitis, and cytomegalovirus (CMV) pneumonitis at 6 months of age. By 8 months, chronic diarrhea and failure to thrive were associated with adenovirus gastroenteritis and frequent hospitalizations for exacerbations of pulmonary disease. Lymphadenopathy and hepatosplenomegaly became increasingly apparent during the second year of life, and by the third year he developed frequent otitis media, gingivitis, and chronic mandibular inflammation. While serum IgM and IgG levels were normal, his IgA level was low. The absolute number of peripheral T cells was reduced, with a significant reduction in $CD4^+$ T cells. In vitro assays demonstrated reduced proliferation after stimulation by anti-CD3 (11 percent of control), PHA (20 percent of control), and other mitogens. Addition of exogenous IL-2 did not significantly rescue these proliferative responses. An allogenic skin graft from a healthy donor (negative for EBV, CMV, HIV, and hepatitis B) was not rejected. Biopsies of lung and upper gastrointestinal tissue revealed dense lymphocytic infiltrates in the absence of infectious agents. Thus, the presentation was of CID with increased susceptibility to viral, bacterial, and fungal infections and evidence of dysregulated lymphocytic inflammatory disease.

The lymphadenopathy and tissue infiltrates of the CD25-deficient patient were partially ameliorated by the administration of corticosteroids. However, to effect a cure an allogeneic bone marrow transplant was performed, using an HLA-matched unaffected sibling as the donor. Engraftment was rapid and a complete resolution of symptoms ensued. On follow-up, the patient was free of symptoms without any treatment with over 95 percent of peripheral blood mononuclear cells of donor origin, and growth and development were appropriate for age.

ABSENCE OF IL2Rα (CD25) EXPRESSION AND MOLECULAR ANALYSIS

EBV-transformed cells from these patients did not express detectable CD25 by flow cytometry, whereas EBV-transformed lines from normal individuals typically contain 20 to 40 percent CD25-positive cells, and Western blot analysis of lysates from peripheral blood lymphocytes confirmed the absence of CD25 protein. In contrast, expression of γc was normal and of IL2Rβ elevated, possibly reflecting compensation for the lack of the α chain, CD25.

Both isoforms of CD25 mRNA had a 4-bp frameshift deletion at cDNA 60–64, resulting in a translational frameshift after 20 codons. The parents each demonstrated a normal and 4-bp-deleted allele.

Atypically for profound cellular immunodeficiency, the thymus in IL2Rα deficiency was of normal size, although Hassall's corpuscles and a distinct corticomedullary demarcation were absent (Plate 10.1). Whereas immunohistochemical staining of thymus sections showed normal expression of CD3, CD2, CD4, CD8, and class I and class II major histocompatibility complex (MHC) proteins, staining for CD1 was completely negative (Plate 10.1). In the normal thymus CD1a is highly expressed on cortical thymocytes and is downregulated upon progression into the medullary antigen-presenting region. Because the *CD1A* gene itself was normal, a failure to provide appropriate signals for CD1 upregulation in thymocytes may explain this phenomenon. CD1 may mediate interactions between thymocytes and thymic epithelium, and absence of CD1 may be related to the loss of the distinct corticomedullary boundary. Normally the induction of CD1 expression in cortical thymocytes precedes a dramatic decrease in the cellular level of the Bcl-2 protein, sensitizing them to undergo apoptosis required for thymic selection (Alvarez-Vallina et al., 1993; Fujii et al., 1994; Gratiot-Deans et al., 1994; Vanhecke et al., 1995).

Whereas normal thymocytes demonstrated distinct areas of low (cortical) and high (medullary) Bcl-2 expression, staining of patient thymus sections revealed that all CD25-deficient thymocytes expressed high levels of Bcl-2 (Plate 15.1). Thus the lack of regulation of Bcl-2 expression in CD25 deficiency suggests dependence upon downstream events following either CD1 or CD25 signaling. Sakaguchi et al. (1995) reported that $CD25^+$ $CD4^+$ regulatory T cells maintain self-tolerance in mice by suppressing autoreactive cells. Indeed, even in the absence of thymic irregularities, autoreactive responses emerged in the absence of $CD25^+$ T cells. This finding suggests that the lymphocytic infiltrates observed in human CD25 deficiency could be autoimmune in nature.

The heavy tissue infiltrates of lymphocytes found in a CD25-deficient patient suggested the involvement of autoreactive oligoclonal expansion observed in multiple autoimmune disorders. Further, the higher-than-normal expression of Bcl-2 in the thymus suggested that such autoreactive clones might escape selection due to reduced ability of these cells to undergo apoptosis. Thus IL2Rα appears to be critical for the physiological negative selection of thymocytes. In its absence, autoreactive clones that normally should die by apoptosis fail to do so, and instead they expand and cause damage to peripheral tissues. In conclusion, a combination of at least two events, one intrathymic and another peripheral, may have combined to create the phenotype observed in humans with CD25 deficiency.

IL2Rα-DEFICIENT MICE

Targeted disruption of IL2Rα in the mouse showed that development of T and B cells appeared intact in these animals, including the distribution of thymocyte subpopulations in the

thymus (Willerford et al., 1995). In addition, NK cells also appeared to be normal (Nelson et al., 1998). However, as with IL-2 deficient mice, older IL2Rα knockout mice developed polyclonal expansion of peripheral T cells that highly expressed CD44 but had a low expression of CD62L. Activated B cells present in young animals initially led to hypergammaglobulinemia, but older animals had progressive loss of B cells. The major phenotype was autoimmune disease, including hemolytic anemia, severe colitis (reminiscent of human ulcerative colitis), and lymphadenopathy.

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11.

T-CELL RECEPTOR COMPLEX DEFICIENCY

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CONCISE DESCRIPTION AND BRIEF HISTORICAL OVERVIEW

Mature T lymphocytes detect the presence of antigens by way of a variable surface heterodimer (either αβ or γδ) termed the T-cell receptor (TCR, Fig. 11.1). In humans, TCR molecules form a complex with two invariant heterodimers called CD3γε and CD3δε and a single invariant homodimer termed CD247 (also called ζζ) (Call et al., 2002). These invariant proteins participate in assembly and surface expression of the whole TCR complex, and in the delivery of intracellular signals that drive T-cell maturation or apoptosis in the thymus, and T-cell activation, proliferation, and effector function or anergy/apoptosis after antigen recognition (Malissen et al., 1999). During early T-cell development, other invariant chains such as the pre-TCR may assist immature TCR ensembles. CD3 and CD247 chains lack intrinsic enzymatic activity for signal transduction. Rather, they relay on conformation- and phosphorylation-dependent recruitment and activation of a number of cytosolic and transmembrane protein tyrosine kinases (PTK) and adaptors such as Zap-70, Fyn, Lck, TRIM, LAT, SLP-76, SIT, and Nck (Schraven et al., 1999). Most TCRαβ-bearing T cells recognize processed peptides associated with major histocompatibility complex (MHC) molecules, whereas the ligands of TCRγδ-bearing T cells are still debated, but include unprocessed bacterial phosphoantigens in humans (Hayday, 2000).

Because of the central role of T cells in adaptive immune responses and the central role of the TCR complex in T-cell selection and function, the description in 1986 of a human familial CD3 expression deficiency in a child with immunodeficiency, but also in his healthy sibling, was in many ways surprising (Regueiro et al., 1986). Four years later, a second CD3 expression deficiency was reported in a healthy child (Thoenes et al., 1990). As it turned out, the former was due to a complete CD3γ deficiency (Arnaiz-Villena et al., 1992) and became the first primary TCR complex immunodeficiency for which the genetic basis was elucidated, while the latter was caused by a partial CD3ε deficiency (Soudais et al., 1993). Further CD3, CD247, and TCR deficiencies followed (Table 11.1), which, keeping with the initial observations, can be classified as complete or partial (also termed leaky) according to the absence or presence of residual levels of the affected protein.

TCR complex deficiencies in humans are very rare autosomal recessive diseases characterized by a selective TCR complex expression defect frequently associated with peripheral blood T, but not B or natural killer (NK), lymphocytopenia and severe combined immunodeficiency disease (SCID) symptoms. TCR complex deficiencies are caused by a range of severe or leaky mutations in the genes encoding for TCR complex chains (to date other than TCRβ, TCRγ or TCRδ). Mutation databases have been established for most of them (http://bioinf.uta.fi/base_root/index.php), as well as diagnostic support websites (http://bioinf.uta.fi/IDdiagnostics).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Reported cases of TCR complex deficiencies have steadily grown to close to 31 patients in 17 families (see Table 11.1), half of them CD3δ deficiencies. Age of onset is generally within the first year of life, essentially with SCID features such as recurrent respiratory infections, chronic diarrhea, and failure to thrive. Chronic pyogenic infections, dysmorphic features, or bone abnormalities were not reported. Unless hematopoietic stem cell transplantation is performed, most

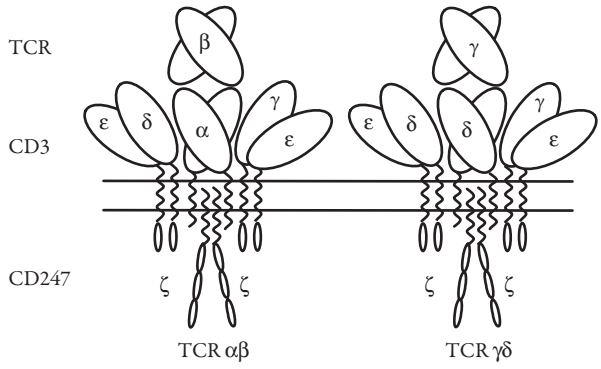

Figure 11.1 *TCR complex isotypes.* Variable TCR heterodimers bind antigens, while invariant CD3 heterodimers (γε and δε) and CD247 homodimers (also called ζζ) undergo conformational changes and recruit intracellular enzymes (such as Fyn, Lck, and Zap) to initiate signal transduction.

patients die early in life as a consequence of viral infections. Omenn syndrome features (hypereosinophilia, hyper-IgE, dermatitis) have been reported in partial CD3δ, CD247, or TCRα defects. In a few cases, notably in complete CD3γ deficiency and in a partial CD3ε deficiency, certain individuals do not show features of immunodeficiency and have reached their third decade in good health without intervention.

LABORATORY FINDINGS

The most consistent laboratory finding is a selective T lymphocytopenia. It may be severe ($T^-B^+NK^+$ immunophenotype), as observed in complete CD3ε or CD3δ defects, with less than 2% peripheral blood T cells, or mild ($T^{+/-}B^+NK^+$), as observed in complete CD3γ or CD247 defects and in most partial TCR complex defects (with >20% T cells, Table 11.2). Overall lymphocytopenia (<3,000 cells/μL in children) is common in the former group, although exceptions due to compensatory B and NK expansions have been reported.

T-lymphocyte functions (anti-CD3 or phytohemagglutinin responses) and B-lymphocyte functions (antibody production following infection or vaccination) are absent when no T cells are detected, although Ig levels may be normal. These functions may be preserved or even normal in partial defects. Autoimmunity and/or immune dysregulation laboratory features may be present, particularly in such leaky defects (see information about Omenn syndrome above).

When T lymphocytes are present, the following laboratory findings have been reported:

1. A TCR complex expression defect is always observed, with 2- to 100-fold less TCR on patient versus normal control T cells using standard CD3ε-specific monoclonal antibodies. It may be severe (more than 10-fold), as observed in CD247 or (partial) CD3ε defects, or mild (less than 5-fold), as observed in CD3γ or (partial) CD3δ or TCRα defects. Thus, a different hierarchy for invariant chain dependence can be proposed for T-cell selection (CD3ε ≥ CD3δ > CD3γ ≥ CD247, see above) as compared with TCR complex expression when some T cells are selected (CD3ε ≥ CD247 > CD3δ ≥ CD3γ). This suggests differential signaling versus structural roles of the different chains during T-cell development.
2. Both αβ and γδ T cells can be detected, but with a restricted repertoire, with few qualifying as recent thymus emigrants (measured using TCR Rearrangement Excision Circles or $CD45RA^+CD27^+$ T cells). However, notable exceptions have been observed, such as partial CD3δ and TCRα defects, which show a $Tαβ^{τ}Tγδ^+B^+NK^+$ immunophenotype with a fairly normal γδ T-cell compartment (Morgan et al, 2010, Gil et al, 2011).
3. In rare cases, two T-cell populations are detected: one with impaired TCR complex expression and a second with normal TCR complex expression (Rieux-Laucat et al., 2006). Somatic mutations that reverted to wild type in certain T-cell clones were found to explain these findings (see the section on mutations analysis below).

Table 11.1 **TCR COMPLEX DEFICIENCIES**

TCR COMPLEX DEFICIENCIES				REFERENCES[a]		NUMBER OF	
PROTEIN	GENE	CHR.	OMIM	COMPLETE	PARTIAL	FAMILIES	PATIENTS
CD3γ	*CD3G*	11	186740	1–5[c]		4	7
CD3δ	*CD3D*	11	186790	6–9	10	7	16
CD3ε	*CD3E*	11	186830	7	11	2	4
CD247[b]	*CD247*	1	186780	12	13	2	2
TCRα	*TRAC*	14	186880		14	2	2
					Total	17	31

[a] [1] Arnaiz-Villena et al., 1992;[2] Sanal, 1996;[3] van Tol et al., 1997;[4] Allende, 2000;[5] Recio, 2007;[6] Dadi, 2003;[7] de Saint Basile et al., 2004;[8] Takada, 2005;[9] Marcus et al., 2011;[10] Gil et al., 2011;[11] Soudais, 1993;[12] Roberts, 2007;[13] Rieux-Laucat et al., 2006; [14] Morgan, 2011.

[b] Also known as TCRζ or CD3ζ

[c] Two new adult patients with CD3γ deficiency and autoimmune rather than immunodeficiency features have been recently published as follows. Tokgoz H, Caliskan U, Keles S, et al. Variable presentation of primary immune deficiency: Two cases with CD3γ deficiency presenting with only autoimmunity. *Pediatric Allergy and Immunology* 2013;24:257–262.

MOLECULAR BASIS

The lack of any invariant TCR complex chain has a profound impact on αβ TCR, pre-TCR, and γδ TCR expression and function. As these receptors are required for T-cell development, T lymphocytopenia ensues in patients, and adaptive immunity is impaired. Different invariant chains show different effects on T-cell selection, as shown in Figure 11.2, supporting the hierarchy indicated above (CD3ε ≥ CD3δ > CD3γ ≥ CD247). TCRα strictly associates to CD3δε dimers, whereas TCRβ has been shown to interact with γε as well as δε dimers before CD247 associates to the TCR complex (Call et al., 2002). This may explain the differential effect of the lack of CD3δ or ε, as compared to CD3γ (or CD247), on T-cell development, which is blocked in complete CD3δ or ε deficiency, but only impaired in human CD3γ or CD247 deficiency.

Table 11.2 TCR COMPLEX DEFICIENCIES: CLINICAL AND IMMUNOLOGICAL DATA

CD3γ

Family	*1*		*2*	*3*	
Nationality	Turkey			Spain	
Patient/sex	P1 M	P2 M	P3 M	P4 M	P5 M
Consanguineous?	YES			NO	
Mutation	Early protein truncation (EPT)				
Diagnosis at (m)	3	7	48	12	11
Present age[1]	†9 m	†20 m	18 y	†32 m	28 y
BMT[2]	No	ID	No	No	No
Lymphopenia (% T cells)	29	39	40	35	43
Cause of death[3]	Sepsis	Pneumonia	AW	Pneumo-nia	AW

[1] 2009 †=exitus at; y (years); m (months)

[2] ID (HLA-matched sibling)

[3] AW (alive and well)

CD3ε

Family	*1*		*2*	
Nationality	French			
Patient/sex	P1 M	P2 F	P3 M	P4 F
Consanguineous?	NO	YES		
Mutation (leaky)	Exon 7 skipping (EPT)	Early protein truncation		
Diagnosis at (m)	24	?	1	birth
Present age[1]	20 y	†5 m	†3 m	†2 m
BMT[2]	No	No	No	H
Lymphopenia (% T cells)	63%	?	?	<1%
Cause of death[3]	AW	Pneu-monitis	CMV	ADV

[1] 2009 y (years); m (months); ND (not done)

[2] H (haploidentical)

[3] AW (alive and well); ADV (adenovirus); CMV (cytomegalovirus)

CD3δ

Family	*1*					*2*	*3*	*4*		*5*	*6*
Nationality	Canada Mennonites					France		Japan		Ecuador	
Patient/sex	1 F	2 M	3 M	4 F	5?	6 F	7 F	8 M	9 F	10 M	11 M	12 M
Consanguineous?	YES					NO						
Mutation	Early protein truncation (exon 2/3)								Exon 3 skipping		Exon 2 skipping	
Diagnosis at (m)	0	2	2	?	?	3	0	5	3	0	14	4
Present age[1]	8 y	†2 m	†3 m	>17 y[4]	?	†5 m	†6 m	†6 m	†3 m	3 y	19 m	†5 m
BMT[2]	MUD	No	No	MUD	MUD	No	H	H	MUD	CB	H	MUD
Lymphopenia (% T cells)	0.1–0.6%			?	?	<1%		0%	1.7%	0.1%	14%	30%
Cause of death[3]	AW	ADV	CMV	AW	AW	CMV	Asperg	EBV	CMV	AW	AW	CMV?

[1] y (years); m (months)

[2] MUD (marrow unrelated donor); H (haploidentical); CB (cord blood)

[3] AW (alive and well); ADV (adenovirus); CMV (cytomegalovirus); EBV (Epstein-Barr virus); Asperg (Aspergillus)

[4] Had a healthy baby in 2008

(continued)

Table 11.2 (CONTINUED)

	CD247		TCRα	
Family	*1*	*2*	*1*	*2*
Nationality	Caribbean	Hawaii	Pakistani	
Patient/sex	P1 M	P2 F	1 F	2 M
Consanguineous?	?	NO	YES	
Mutation (*leaky*)	Early truncation	Late insertion	Exon 3 skipping	
Diagnosis at (m)	4	10	15	6
Present age	8 y	10 y	?	?
BMT	Haploidentical	Haploidentical	Haploidentical	Haploidentical
Lymphopenia (% $CD3^{dull}$ T cells)	4–17%	63%	21%	50%
Cause of death	Alive & well	Alive & well	Alive & well	Alive & well

FUNCTIONAL ASPECTS

TCR complex function obviously cannot be studied in patients with TCR complex defects that block T-cell development. When some T cells are present, meaningful comparisons with normal individuals are difficult because T-cell subset representation and surface TCR complex expression are altered. Nonetheless, it is clear that normal TCR signaling is possible in vivo, since selection took place in those patients and in some cases (CD3γ, partial CD3ε) normal antibody responses indicate intact helper T-cell functions. T-cell lines from patients have been difficult to derive. Our studies in human CD3γ-deficient primary T cells, interleukin (IL)-2-dependent T-cell lines, and Herpesvirus saimiri- or HTLV-I-transformed T lymphocytes indicated that CD3γ contributes to but is not required for the regulation of TCR trafficking in resting and antigen-stimulated mature T lymphocytes (Torres et al., 2003). Despite its effects on TCR complex expression (likely due to impaired recycling), CD3γ is dispensable for several TCR-induced mature T-cell responses, such as calcium flux, cytotoxicity, up- or downregulation of several surface molecules, and proliferation and synthesis of certain cytokines (TNFα). In contrast, phorbol myristate acetate-induced TCR complex downregulation and TCR-induced synthesis of other cytokines (IL-2) as well as adhesion and polarization were severely impaired (Arnaiz-Villena et al., 1992; Pacheco-Castro et al., 1998; Perez-Aciego et al., 1991; Torres et al., 2002). The lack of CD3γ causes a stronger impairment of αβTCR expression in $CD8^+$ than in $CD4^+$ T cells in humans and in mice. We have shown that this is due to biochemical differences in the intracellular control of αβTCR complex assembly, maturation, or transport between the two lineages, which result in conformational lineage-specific differences regulated by activation or differentiation both in normal and in CD3γ-deficient primary T cells (Zapata et al., 1999, 2004). More recently, we have reported that the lack of CD3γ in humans caused a stronger impairment of CD3 expression in αβ than in γδ T cells (Siegers et al., 2007), whereas the opposite is true in partial CD3δ deficiency (Gil et al, 2011).

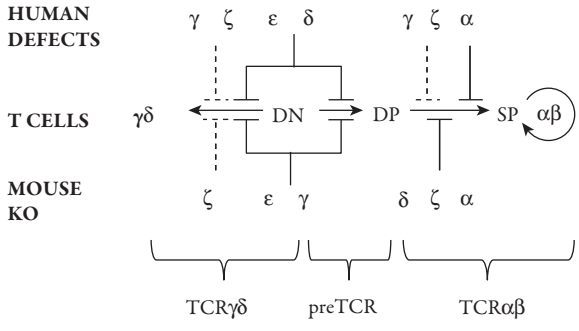

Figure 11.2 Leaky (dashed) or severe (solid) block of early T-cell differentiation caused by complete invariant TCR complex chain defects in humans or mice. αβ T-cell development is simplified in two steps: (1) pre-TCR-mediated double-negative (DN) $CD4^-CD8^-$ to double-positive (DP, $CD4^+CD8^+$) transition and (2) αβ TCR-mediated positive/negative selection and generation of single-positive (SP) $CD4^+$ and $CD8^+$ αβ T cells. γδ T cells develop from DN thymocytes. CD247 is depicted as ζ for brevity.

MUTATION ANALYSIS

Mutation analysis was started by probing T-cell RNA with *CD3*, *CD247*, or *TRAC*-specific sequences. For some CD3δ defects, microarray analysis of thymocyte RNA revealed low specific transcript levels. In all cases, cDNA was synthesized and used to amplify and sequence TCR complex genes. This revealed the presence of point mutations or small deletions (Fig. 11.3), which could be traced with mutation-specific oligonucleotides, restriction enzymes, or direct sequencing. Small deletions were due to splicing site mutations, which were identified on genomic DNA by sequencing relevant exon boundaries. As a consequence, no or very few specific proteins of the TCR complex could be detected biochemically.

In a partial CD247 deficiency, reversion of some T-cell clones to normal expression was observed in vivo as a consequence of additional mutations in T-cell precursors (Rieux-Laucat et al., 2006).

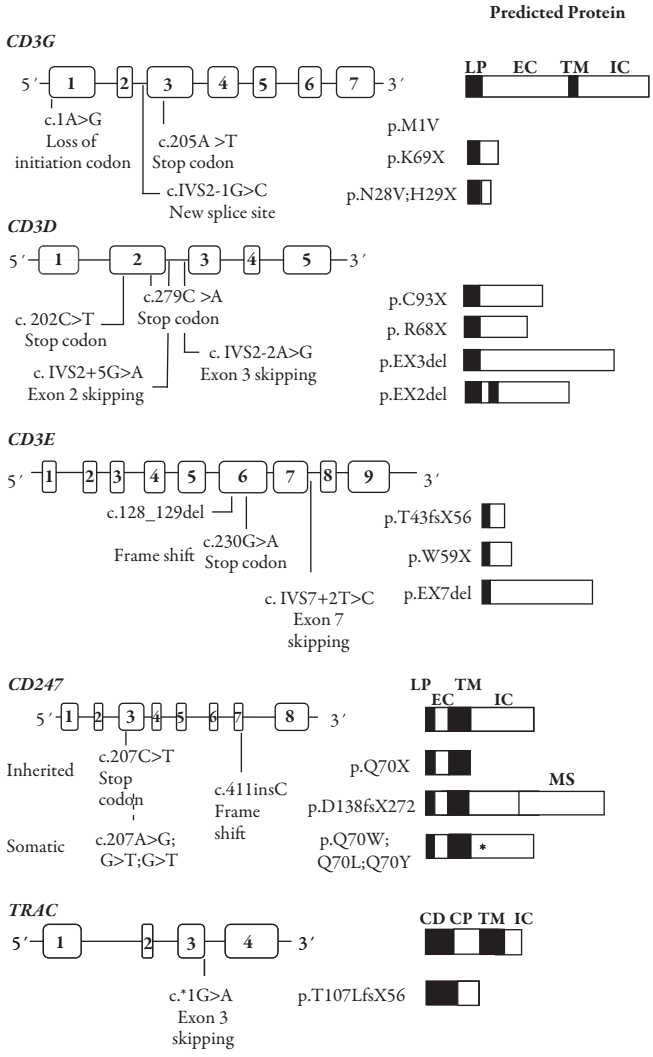

Figure 11.3 Mutations reported in genes encoding for TCR complex chains and predicted proteins. LP, leader peptide; EC, extracellular; TM, transmembrane; IC, intracellular; CD, constant domain; CP, connecting peptide; UT, untranslated.

STRATEGIES FOR DIAGNOSIS

Definitive: Male or female patient with surface TCR complex expression defect, selective peripheral blood T lymphocytopenia ($T^-B^+NK^+$ or $T^{+/-}B^+NK^+$ phenotype), and mutations in a TCR complex gene (such as *CD3G, CD3D, CD3E, CD247*, or *TRAC*).

Probable: Male or female patient with surface TCR complex expression defect and selective peripheral blood T lymphocytopenia ($T^-B^+NK^+$ or $T^{+/-}B^+NK^+$ phenotype)

Spectrum of disease: From SCID (common) to healthy (rare, overlooked?). Complete CD3ε or CD3δ defects show the $T^-B^+NK^+$ phenotype, whereas complete CD3γ or CD247 defects and partial defects tend to show the $T^{+/-}B^+NK^+$ phenotype. T-cell revertants with normal TCR complex expression due to somatic mutations may be present.

Differential diagnosis: With patients showing $T^-B^+NK^+$ or $T^{+/-}B^+NK^+$ phenotypes, such as those with defects in IL7Rα, FOXN1, Coronin-1A, Zap70, MHC class I or II, PNP, ADA, or DiGeorge syndrome

Testing for the percentage of $CD3^+$ lymphocytes may not be enough to detect TCR complex deficiencies, particularly when some T cells are present. Analyzing the mean fluorescence intensity is mandatory, as well as using a range of TCR-, CD3-, and CD247-specific monoclonals. The expression defect follows the CD3ε ≥ CD247 > CD3δ ≥ CD3γ hierarchy with a wide fold-difference range.

Biopsy specimens from lymphoid tissues should be thoroughly studied (Arnaiz-Villena et al., 1991; Dadi et al., 2003; Morgan et al., 2011) and T cells preserved if possible (Pacheco et al., 1998; Perez-Aciego et al., 1991) and analyzed by immunoprecipitation (Perez-Aciego et al., 1991; Thoenes et al., 1992) and molecular biology techniques (Arnaiz-Villena et al., 1992; Soudais et al., 1993).

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

TCR complex deficiencies are autosomal recessive disorders. Heterozygotes are healthy and cannot be easily distinguished from normals by standard laboratory tests, although half-normal CD3 expression levels have been reported by flow cytometry (Brooimans et al., 2000; Muñoz-Ruiz et al., 2013) or biochemistry (van Tol et al., 1997). Thus mutation analysis must be performed in each case, as explained above. Restriction fragment length polymorphism (RFLP) analysis using TaqI and a *CD3E* probe (50% heterozygosity) or polymorphic markers may help to define *CD3GDE* haplotype inheritance for carrier detection and/or prenatal diagnosis, since recombination within the *CD3* gene complex is rare.

TREATMENT AND PROGNOSIS

Unless the patient is transplanted, the prognosis is very poor for those with complete defects except CD3γ and for most partial defects (see Table 11.2). Matched related, haploidentical mismatched related (MMRD), matched unrelated (MUD), and mismatched unrelated donors have all been used for hematopoietic stem cell transplantation, with bone marrow, peripheral blood, or cord blood as sources. The recipients generally underwent myeloablative conditioning. The largest series consisted of patients with CD3δ defects; they showed a superior outcome using MUD as compared to MMRD (Marcus et al, 2011). Viral infections (herperviruses) are the most common cause of death among transplanted patients. Successfully transplanted patients have been shown to lead a normal life up to 18 years posttransplantation.

A few patients had no immunodeficiency symptoms and thus did not receive hematopoietic stem cell transplantation (CD3γ, partial CD3ε), reaching their third decade in good health. In those cases prophylactic intravenous immunoglobulin (IVIG) with (Le Deist et al., 1991) or without (van Tol et al., 1997) antibiotics were used, or antibiotics only when symptoms developed (Allende et al, 2000). The observation

that most antibody responses were normal in vivo in one case prompted a comprehensive vaccination program, excluding attenuated live viruses. No secondary effects were recorded. Thus, this approach may be helpful for other TCR complex-deficient patients on a preventive basis. Bronchial asthma in one case was treated with ketotifen and cromolyn sodium between 3.5 and 7 years of age (Sanal et al., 1996), followed by salbutamol sulfate and sodium chromoglycate to manage his nonatopic hyperreactive airway, including eformoterol with occasionally inhaled steroids. Gene therapy protocols were tested in vitro (Sun et al., 1997). However, transfer of CD3γ into mature T cells may disrupt their intrathymic fine tuning (Pacheco-Castro et al., 2003). Thus, lymphoid progenitors may be better targets in this case, although the selective advantage of transduced over untransduced T cells remains to be established.

ANIMAL MODELS

Single as well as multiple TCR complex deficiencies have been created in mice through gene targeting (Malissen et al., 1999; Mombaerts et al., 1992). Ablation of any invariant TCR complex protein essentially blocked T-cell development, although at different intrathymic checkpoints, and to a different extent (see Fig. 11.2). Indeed, all invariant TCR complex proteins, except CD3δ, are required for T-cell selection at the pre-TCR (TCRβ) checkpoint, with the following hierarchy: CD3ε > CD3γ > CD247. However, all invariant TCR complex chains, including CD3δ, are required for T-cell selection at the TCRαβ checkpoint and for αβTCR surface expression. Interestingly, CD3δ is also dispensable for γδ T-cell selection and for γδTCR surface expression in mice, but not in humans (Dadi et al., 2003). This is due to a differential stoichiometry of the γδTCR between the species (Siegers et al., 2007). The mouse surface γδTCR does not incorporate the CD3δ subunit; thus, its stoichiometry is TCRγδCD3εγεγζζ rather than TCRγδCD3εδεγζζ, as observed in humans (see Fig. 11.1). The murine models are similar to human CD3 deficiencies in some aspects (ε > γ in αβTCR expression, no peripheral T cells when CD3δ is lacking) but not in others (peripheral blood T-lymphocyte numbers are clearly higher in humans lacking CD3γ). Thus, peripheral lymphoid expansion mechanisms may differ between species. CD3 gene inactivation in mice, even when kept in pathogen-free facilities, may cause pathological manifestations, including enteropathy in ζ/η- or CD3δ-deficient mice, which resemble those observed in some CD3γ- or CD3δ-deficient humans.

CONCLUDING REMARKS

The TCR complex is first expressed and used by T cells early during their intrathymic development. Accordingly, complete TCR complex deficiencies strongly impair early T-cell differentiation events in humans, generally causing SCID. TCR complex deficiencies provide insights into the redundant and unique roles of these transmembrane molecules for TCR complex assembly and signal transduction and thus for T-cell selection and antigen recognition, which are not always recapitulated by murine models.

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12.

SEVERE COMBINED IMMUNODEFICIENCY DUE TO MUTATIONS IN THE *CD45* GENE

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The protein tyrosine phosphatase CD45, also known as protein tyrosine phosphatase receptor type C (PTPRC) and leukocyte common antigen (LCA) (MIM 151460; disease #608971), is an abundant, high-molecular-weight type I transmembrane glycoprotein that is exclusively expressed on all nucleated hematopoietic cells (Thomas, 1989; Tonks et al., 1988; Trowbridge and Thomas, 1994). CD45 regulates the activity of receptor-associated Src-type tyrosine kinases (Alexander, 2000; Ashwell and D'Oro, 1999; Hermiston et al., 2002; Penninger et al., 2001; Thomas, 1999; Thomas and Brown, 1999). Its absence is associated with a block in signaling via antigen receptors (Desai et al., 1994; Koretzky et al., 1990; Pingel and Thomas, 1989). CD45 also downregulates the function of integrin-mediated adhesion and cytokine receptor signaling by dephosphorylating integrin-associated Src kinases (Roach et al., 1997) and cytokine receptor-associated Jak kinases, respectively (Irie-Sasaki et al., 2001).

CD45 deficiency in humans results from rare deleterious point mutations and deletions that are inherited in an autosomal recessive manner. Two patients have been described who presented in infancy with severe combined immunodeficiency (SCID) associated with T-cell depletion and failure of the residual T cells in the periphery to respond to mitogenic stimuli (Cale et al., 1997; Kung et al., 2000; Tchilian et al., 2001). The B cells, whose development was spared, failed to develop germinal centers or to sustain normal immunoglobulin production. The immunological abnormalities of these patients mirrored those of animal models of CD45 deficiency (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999), reflecting a requirement for CD45 in antigen receptor signaling during T-cell development and upon T- and B-cell activation in the periphery. A third, unrelated patient, has been reported who had a similar phenotype due to having inherited 2 maternal copies of a CD45-mutation-bearing chromosome 1, with no paternally derived chromosome 1; this situation is known as maternal uniparental isodisomy of chromosome 1 (Roberts et al., 2012).

REGULATORY FUNCTIONS OF CD45

The *CD45* gene, located on chromosome 1q31.3-q32.1, contains 34 exons encoding multiple splice variants (Hall et al., 1988). The extracellular portion of CD45 has a heavily *O*-glycosylated amino-terminal region and a cysteine-rich region containing fibronectin type III sequence motifs. Cell type-specific alternative splicing of exons 4/A, 5/B, and 6/C generates up to 8 CD45 isoforms, encoding extracellular regions ranging from 391 to 552 amino acids (Rogers et al., 1992). Differential interaction of these with other proteins either in *cis* or in *trans* may influence cell migration and alter biochemical signaling regulated by CD45.

In T cells, different CD45 isoforms are expressed in a manner specific to developmental stage and activation state (reviewed in Trowbridge and Thomas, 1994). Immature thymocytes express low-molecular-weight CD45 isoforms. Naïve T cells express high-molecular-weight, CD45RA-containing isoforms. Following activation, T cells switch to express a lower-molecular-weight isoform lacking all three alternatively spliced exons (CD45RO). The CD45RO phenotype found upon memory T cells is reversible in that CD45RO T cells can later resume expression of the high-molecular-weight isoforms. B cells express the largest CD45 isoform of 220 kD molecular weight (also known as B220) containing all 3 alternative exonic sequences (CD45RABC).

The CD45 cytoplasmic region contains two prototypic phosphatase domains in tandem. The proximal domain is catalytically active, whereas the distal pseudophosphatase domain is inactive but promotes recruitment of substrate proteins (Kashio et al., 1998). Reconstitution of the cytoplasmic domain of CD45 as part of chimeric molecules restores signaling via the T-cell antigen receptor in CD45-deficient cell lines, indicating that the lack of CD45 phosphatase activity is the fundamental abnormality underlying the failure of antigen receptor signaling in CD45 deficiency (Desai et al., 1994).

The role of CD45 in lymphocyte antigen receptor signaling centers on regulating the activity of antigen receptor-associated Src family kinases: p56Lck and p59Fyn in T cells, and Lyn in B cells (Thomas and Brown, 1999). A major function of CD45 is to maintain these kinases in a quiescent state, yet poised for activation upon engagement of antigen receptors. The activity of Src kinases is regulated by the phosphorylation status of 2 tyrosine residues: one at the carboxyl terminus, which serves a negative regulatory function; and the other within the kinase domain, which serves a positive regulatory function. In CD45-deficient cells, the C-terminal regulatory tyrosine residue is consistently found hyperphosphorylated, indicating that CD45 functions to maintain this site in a dephosphorylated form. Phosphorylation of the C-terminal regulatory tyrosine residue results in its intramolecular association with the SH2 domain of the same Src kinase, which locks the kinase in a closed conformation that renders it inactive. The deleterious impact of hyperphosphorylation Src kinase C-terminal inhibitory tyrosine in CD45 deficiency is highlighted by the observation that a constitutively active Lck mutant, in which the C-terminal tyrosine residue is changed into a phenylalanine, rescues the profound block in T-cell development in CD45-deficient mice (Pingel et al., 1999; Seavitt et al., 1999).

The positive regulatory tyrosine residue in the catalytic domain of Src kinases also serves as a substrate for CD45. This role is particularly important in regulating the function of Lck activity in thymocyte development and in downregulating the activity of the Src kinases Hck and Lyn during integrin-mediated adhesion in macrophages.

Given that the net effect of CD45 is to maintain Src kinases in a dephosphorylated, yet primed state, how are the positive effects of CD45 on antigen receptor signaling mediated? One answer to this question was provided by the finding that CD45 is excluded from the immunological synapse, the supramolecular structure formed at the interface of T cells with antigen-presenting cells that includes the T-cell receptor (TCR), CD4 and CD8 co-receptors, antigen-presenting major histocompatibility complex (MHC) molecules, and a variety of signaling molecules (Johnson et al., 2000). Lipid rafts containing the TCR, Lck, and the adapter protein LAT but excluding CD45 cluster at the immunological synapse, allowing effective src kinase activation.

A second mechanism by which CD45 activity is regulated involves receptor dimerization. On the basis of crystal structure of a related phosphatase, RPTPα, it was predicted that the juxtamembrane and proximal catalytic domains of CD45 form dimers in which the catalytic site of one catalytic domain is blocked by a wedge formed by the juxtamembrane region of its partner. Consistent with this model, mice carrying a mutation of the conserved glutamic amino acid to arginine at the tip of the CD45 wedge domain develop lymphoproliferation and autoimmune nephritis, as also associated with hyperactive src kinases (Majeti et al., 2000). The different CD45 isoforms may vary in their capacity to form dimers (Hermiston et al., 2002). According to this hypothesis, the extensive *O*-linked glycosylation and sialylation found on the alternatively spliced exons 4, 5, and 6 render the large CD45RA isoforms expressed on naïve T cells more resistant to dimerization because of electrostatic repulsion. In contrast, the absence of this electrostatic barrier in CD45RO memory T cells promotes dimerization. This may explain the more effective signaling in memory T cells.

While CD45 promotes signaling via antigen receptors, it can dampen signaling by other receptor complexes. For example, CD45 negatively regulates integrin-mediated adhesion, an effect related to its ability to dephosphorylate Src kinases at integrin focal adhesion sites (Roach et al., 1997). Consistent with this function is the observation that CD45-deficient T cells and macrophages are abnormally adherent, a phenotype that is reversed by reconstitution of CD45 expression. As in the case of lymphocyte antigen receptor, a topological explanation underlies the regulatory function of CD45 in integrin-mediated adhesion (Thomas and Brown, 1999). Unlike the case of signaling via antigen receptors, where CD45 is excluded from the immunological synapse, CD45 co-localizes with integrin-centered focal adhesion sites. This serves to maintain Src kinases clustered at these sites under negative dephosphorylation pressure.

CD45 also exerts negative regulation upon signaling via cytokine receptors, where it acts to dephosphorylate receptor-associated Jak kinases (Irie-Sasaki et al., 2001; Penninger et al., 2001). CD45-deficient mice exhibit increased cytokine-dependent myelopoiesis and erythropoiesis and are resistant to otherwise fatal cardiomyopathy associated with Coxsackie virus B3 infection. Given that other molecules, such as SOCS (suppressor of cytokine signaling) and nonreceptor phosphotyrosine phosphatases SHP1 and SHP2, also contribute to negative regulation of Jak kinase activation, the precise role of CD45 in negative regulation of Jak kinase activation remains to be fully mapped.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Of the two original cases of CD45-deficient SCID, one was a boy born to unrelated Finnish parents (Kung et al., 2000) and the other was a girl born to consanguineous Kurdish parents (Cale et al., 1997; Tchilian et al., 2001) (Table 12.1). The Finnish child suffered from recurrent infections starting at 6 weeks of age, including candida skin and oral infections, recurrent otitis media, bronchitis, conjunctivitis, impetigo, gluteal abcess, and chronic rotavirus diarrhea. He had leukopenia, lymphopenia, and severe anemia with marrow erythroplasia

Table 12.1 CLINICAL FINDINGS IN TWO CD45-DEFICIENT PATIENTS

FACTOR	FINNISH	KURDISH
Age at presentation	6 weeks	2 months
Failure to thrive	Yes	Yes
Recurrent bacterial or viral infections	Yes	Not reported
Chronic rotavirus diarrhea	Yes	NR
Opportunistic infections	BCG, oral candidiasis	CMV
Bone marrow transplant	No	Yes
Terminal event	B-cell lymphoma	Reactivated CMV infection

Data from Cale et al. (1997); Kung et al. (2000); Tchilian et al. (2001).

BCG, bacillus Calmette-Guérin; CMV, cytomegalovirus.

Table 12.2 LYMPHOCYTE SUBPOPULATIONS IN HUMAN AND MURINE CD45 DEFICIENCY

	CELL COUNT (% OF PERIPHERAL LYMPHOCYTES)*		
	CD45 KNOCKOUT MICE	FINNISH PATIENT	KURDISH PATIENT
CD3	Depleted	Depleted (8.9%)	Depleted (14.9%)
CD3/4	Depleted	Depleted (0.9%)	Depleted (4%)
CD3/8	Depleted	Depleted (4.9%)	Decreased (20%)**
TCRαβ	Depleted	Depleted (2.2%)	NR
TCRγδ	Preserved	Normal (6.7%)	NR
NK	Preserved	Decreased (3%)	NR
B cells	Preserved	Normal (84.5%)	Normal (68.9%)

*Mice were deficient in CD45 exons 6, 9, and 12 (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). Patients are from Cale et al. (1997); Kung et al. (2000); Tchilian et al. (2001).

**Percentage refers to total $CD8^+$ cells in the periphery.

NR, not reported.

requiring transfusions. Reticulocytes appeared in the blood at the age of 3 months and his hemoglobin levels normalized thereafter, but lymphopenia persisted. Having been vaccinated with bacillus Calmette-Guérin (BCG) at birth, he developed BCG infection at age 4 months and isoniazid treatment was instituted. Despite supportive therapy, he suffered from severe malnutrition and failure to thrive. He had unrelenting chronic diarrhea associated with persistent *Clostridium difficile* and rotavirus infection. Respiratory syncytial virus antigen test was repeatedly positive in his nasopharyngeal aspirate. At the age 1 year and 5 months, weighing 5.5 kg, he was evaluated for bone marrow transplantation (BMT), but he died soon thereafter from an aggressive B-cell lymphoma.

The Kurdish child presented at the age of 2 months with disseminated, postnatally acquired cytomegalovirus (CMV) infection associated with fever, rash, pneumonitis, lymphadenopathy, hepatosplenomegaly, and pancytopenia. She underwent BMT at the age of 8 months. Despite T-cell engraftment she experienced fatal CMV reactivation. The third patient presented at 6 months of age with failure to thrive and gastroesophageal reflux and at age 10 months had *Pneumocystis jiroveci* pneumonia and adenovirus. He had T and B lymphopenia and absent proliferation to PHA. He was rescued with a maternal T cell depleted haploidentical hematopoietic cell transplant, as well as antiviral and antibiotic therapy, at Duke University Medical Center in the U.S.; he survived with excellent T cell engraftment and function, though he still requires immunoglobulin supplementation (Roberts et al., 2012).

LABORATORY FINDINGS

The laboratory findings of human CD45 deficiency were in accord with animal models (Table 12.2), with patients demonstrating lymphopenia, with particularly depleted CD4 T cells. B cells were normal in number, but immunoglobulin levels were low. The natural killer (NK) cell population was decreased in the Finnish infant (3 percent). CD45 expression as determined by flow cytometry was either totally absent (Finnish and U. S. infants) or minimally present (Kurdish infant). Parents of all children had normal CD45 expression. Proliferative responses to mitogenic lectins were totally absent in all patients.

MOLECULAR BASIS

All reported patients suffered from autosomal recessive defects in the CD45 gene (Fig. 12.1). The Finnish infant carried a large deletion of the 3' end of the gene on one allele, while the other had a G-to-A transition at the invariant (+1) position of the donor splice site of intron 13, resulting in no expression of normal mRNA (Kung et al., 2000). The Kurdish infant had a homozygous 6 bp deletion at nucleotide 1168 in exon 11 of the CD45 gene, resulting in deletion of glutamic acid 339 and tyrosine 340 in the first fibronectin type III module of the CD45 extracellular domain (Tchilian et al., 2001). When a mutant CD45 cDNA carrying this 6 bp deletion was transfected into Chinese Hamster Ovary and mouse EL-4 thymoma cells, no protein was found at the cell surface, although it was detected at reduced levels intracellularly. This finding indicated that the deleted amino acids may contribute to the proper folding, stability, and/or correct localization of the mutant protein. The U. S. Duke patient was homozygous for cDNA 1618 A tcT, causing a stop codon at amino acid position 540 (K540X).

STRATEGIES FOR DIAGNOSIS AND TREATMENT

CD45 deficiency has a characteristic picture of T^- B^+ SCID with profound but not total T-cell depletion, absent

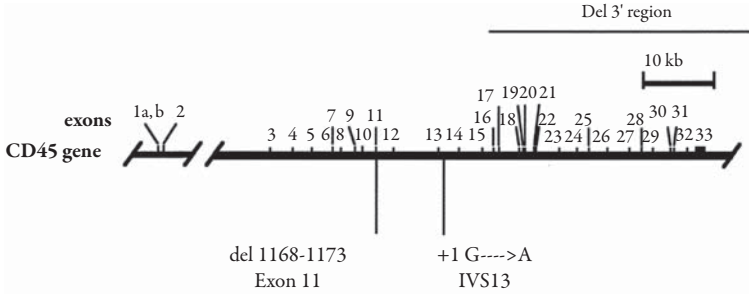

Figure 12.1 Genomic organization of CD45 gene organization. The mutations found in the affected infants are indicated. The Finnish infant suffered from a large deletion in the 3' region of the gene and a G → A substitution in the splice donor junction of intervening sequence (IVS) 13. The Kurdish homozygous 6bp deletion in exon 11, corresponding to nucleotides 1168–1173 of CD45 cDNA. The U. S. patient had a homozygous trunction mutation in exon 15 (not shaer).

proliferative responses to T-cell mitogens, and normal B-cell numbers. Little or no expression of CD45 was found on the surface of peripheral blood mononuclear cells in the cases discussed here. However, it is possible that loss-of-function CD45 mutations might retain expression of mutated protein; in such cases CD45 abnormalities can be excluded by evidence of normal signaling events triggered via TCR/CD3, including calcium mobilization and tyrosine phosphorylation. Alternatively, molecular lesions can be sought by DNA sequencing.

Definitive therapy for CD45 deficiency would be BMT. The fatal outcomes in the two patients reported point to the lethal nature of this immunodeficiency and the urgent need for timely immune reconstitution. As in other cases of SCID, the ideal transplant would be with bone marrow from a human leukocyte antigen (HLA)-matched sibling, followed by bone marrow from matched unrelated donors or HLA-haploidentical family members, as was successful in the Duke case.

ANIMAL MODELS

Studies of three different CD45 knockout mice, targeting exons 6, 9, and 12 respectively, have revealed a critical function for CD45 in thymocyte development (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). The most pronounced defect in the development in CD45-deficient thymocytes involves positive selection at the $CD4^+$ $CD8^+$ stage. This results in the exit into the periphery of only 5 to 10 percent of the expected number of mature T cells. Negative selection is also somewhat impaired. Both defects reflect reduced, though not totally absent, TCR signaling in CD45-deficient thymocytes, leading to a raised selection threshold. A constitutive active p56Lck mutant rescued thymocyte development in CD45-deficient animals, consistent with impaired activation of Lck as the principal underlying mechanism involved in ineffective selection of CD45-deficient thymocytes (Pingel et al., 1999; Seavitt et al., 1999). In the periphery, T-cell activation via the TCR, proliferation, and cytokine secretion in response to TCR ligation were abrogated.

As in humans, B-cell development was spared in CD45-deficient mice. However, B-cell proliferation in response to cross-linking of surface IgM was impaired (Benatar et al., 1996). A heightened signaling threshold also led to defects in positive and negative selection of maturing B cells, with impairment of the transition from IgM^{hi} IgD^{lo} to IgD^{hi} B cells. Negative selection at this stage was also impaired, also a reflection of attenuated BCR signaling (Cyster et al., 1996). T-cell independent B-cell responses were intact.

CONCLUSIONS AND FUTURE DIRECTIONS

The SCID phenotype associated with CD45 deficiency reflects the critical role played by this phosphatase in promoting antigen receptor signaling and in regulating the function of other signaling complexes, including cytokine receptors and integrins. CD45 deficiency expands the spectrum of immunodeficiency diseases associated with phosphotyrosine signaling pathways, including ZAP-70 and IL-7 receptor/common gamma chain/Jak3 kinase deficiency syndromes in T cells and Btk deficiency in B cells. It is plausible that CD45 deficiency represents the extreme end of a spectrum of CD45-related diseases associated with immunodeficiency, autoimmunity, and/or inflammation. Such disorders may result from selective defects in the expression of individual CD45 isoforms or abnormalities in CD45 function related to one or more receptor complexes. In mice, enhanced expression of particular CD45 isoforms in T-cell subsets has been associated with a number of autoimmune diseases (Penninger et al., 2001). In humans, a C/G polymorphism at nucleotide 77 in exon 4/A of CD45 that results in defective alternative splicing of exon 4/A has been associated with multiple sclerosis, although this association was not reproduced by other studies (Barcellos et al., 2001; Jacobsen et al., 2000; Vorechovsky et al., 2001). Future studies will further clarify mechanisms by which CD45 deficiency leads to SCID and the role of CD45 in other immunological diseases.

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13.

V(D)J RECOMBINATION DEFECTS

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The vertebrate cognate immune system recognizes and responds to a virtually infinite number of foreign antigens via antigen-specific immunoglobulin (Ig) or T-cell receptor (TCR) molecules expressed on the cell surface of B and T lymphocytes, respectively. While the Ig receptor of B cells binds to soluble antigens, the TCR receptor recognizes peptide antigens presented by human leukocyte antigen (HLA) molecules. In general, allelic exclusion prevents expression by a single lymphocyte of two different receptors encoded by homologous alleles. The expression of each distinct receptor is maintained in the clonal progeny of a lymphocyte.

V(D)J RECOMBINATION

Immunoglobulin and TCR chains each consist of two structural domains: their constant regions mediate effector functions, while the variable parts of the receptor chains form an antigen-binding pocket. Site-specific recombination events (VDJ recombination) in the receptor genes of each developing lymphocyte lead to generation and expression of a particular variable domain assembled from a set of subgenic segments classified as variable (V), diversity (D), and joining (J) elements (for review see Chapters 4 and 5, and Schlissel and Stanhope-Baker, 1997; Nemazee, 2000). In principle, each of the V elements can join to any of the D and J modules, thus allowing a finite number of subgenes to establish enormous antigen receptor diversity.

Seven gene loci encoding the Ig heavy (IgH) and light chains as well as the TCR α, β, γ, and δ chains can potentially undergo somatic DNA recombination during lymphocyte development. The loci share a similar conserved overall organization (Color Plate 13.IA); however, the precise number of subgenic segments and their organization vary between different loci and species (Litman et al., 1993).

The principle of V(D)J recombination is a simple cut-and-paste mechanism, fusing in each step two subgenic DNA segments (Plate 13.IB) (Lewis, 1994). This reaction is based on a universal tag for all V, (D), and J modules. These gene segments are flanked by recombination signal sequences (RSSs) (Plate 13.IA), which consist of a conserved heptamer and an AT-rich nonamer nucleotide motif, separated by a 12 ± 1 bp or 23 ± 1 bp spacer (Ramsden et al., 1994). Spacer length, therefore, defines two types of RSSs, termed 12-RSS and 23-RSS, and efficient recombination occurs only between a 12-RSS and a 23-RSS, a restriction called the *12/23 rule*. Additional regulatory effects of RSS sequence on recombination have been described (Bassing et al., 2000).

The V(D)J recombination reaction can be divided into two main steps involving several different subreactions (Table 13.1). The first part of the recombination process is lymphocyte-specific and comes about through an endonucleolytic cut in DNA leading to double-strand breaks (DSBs) at the border between the RSS heptamer and the flanking coding segment. In vivo, in a single V(D)J recombination reaction, four DNA ends are generated simultaneously through the synchronous cuts at two distinct gene segments: two blunt, phosphorylated signal ends and two hairpin-sealed coding ends. The second part of the V(D)J rearrangement involves the processing of the signal and coding ends, in which, ultimately, factors of a ubiquitous DNA double-strand repair pathway are recruited to link the signal and coding ends.

The standard products of V(D)J recombination result from ligation of the two coding elements (coding joints) and of the two heptamers of the RSS (signal joints). Depending on the orientation of the two RSSs to each other, the rearrangement process leads to a DNA deletion or inversion

(Plate 13.IC). Most rearrangement events stem from a deletion of the DNA connecting the recombined V(D)J elements and produce an extrachromosomal DNA circle with a signal joint (Fujimoto and Yamagishi, 1987; Okazaki et al., 1987). At the TCRβ, TCRδ, and Igα loci, inversions have been noticed (Feddersen and van Ness, 1985; Malissen et al., 1986; Korman et al., 1989), with a slight preference of deletions over inversions (Hesse et al., 1987). V(D)J recombination can result in alternative products such as "hybrid" or "open-and-shut" junctions (Plate 13.IC). *Hybrid joints* arise through the ligation of one coding end to the signal end of the other. In an *open-and-shut joint*, which is less common, the signal and coding ends created by site-specific cleavage are modified prior to their religation (Lewis, 1994). These alternative products are rare events, but they obey the rules of V(D)J recombination and are indicative of the notion that four open DNA ends are intermediates in the rearrangement process. The signal ends are usually ligated without modification of the DNA (Lieber et al., 1988). In contrast, joining of coding ends is generally imprecise, with base losses and/or additions (N and P nucleotides) of approximately 10 to 15 nucleotides. This process helps to diversify the receptor repertoire but introduces the risk of creating nonfunctional genes because of out-of-frame joining and/or introduction of premature stop codons. The enzyme terminal deoxynucleotidyl transferase (TdT) adds random, GC-enriched nucleotides (N nucleotides) to coding ends by a template-independent polymerization. P nucleotides represent short, palindromic repeats of coding end DNA. They are thought to be generated when the "hairpin," a coding end intermediate of the V(D)J recombination reaction (Color Plate 13.II), is resolved through an endonucleolytic attack, not at the tip of the covalently closed termini but within the coding element. Other mechanisms contributing to junctional diversity are erosion of a small and variable number of bases at coding ends by exo- or endonucleases, and homology joining through short-sequence homologies at free DNA ends (for review see Fugmann et al., 2000a).

The "recombinase" machinery, a multiprotein complex, is responsible for the V(D)J recombination. Nine proteins have been identified thus far as participating in the various steps of the V(D)J recombination reaction (Table 13.1), and the proteins the mediate recombination are listed in Table 13.2. The recombination activating genes 1 and 2 (*RAG1* and *RAG2*) encode proteins necessary and sufficient to initiate V(D)J recombination at an accessible antigen receptor gene locus (McBlane et al., 1996). The second step of this reaction requires the Artemis protein encoded by the gene *DCLRE1C* in addition to RAG1/RAG2 processing of the coding end intermediates. The template-independent DNA polymerase TdT adds N nucleotides and contributes substantially to receptor diversity. The DNA-dependent protein kinase (DNA-PK), with its DNA binding constituents KU70 and KU80 and its catalytic subunit DNA-PKcs, recognizes open DNA ends. XRCC4 (the gene product responsible for the defect of cells of group 4 of X-ray cross-complementing cell lines) seems to interact with Ligase IV in the ligation step of DNA DSB repair. *Cernunnos/XLF*, a recently identified NHEJ gene structurally related to XRCC4, participates in the same complex together with Ligase IV. Despite this recent progress, many different factors of the recombinase machinery (*trans-acting* factors for locus accessibility and exo- and endonucleases) are still unknown. Of the identified factors, the *RAG1*, *RAG2*, *LIG4*, *DCLRE1C (Artemis*), *NHEJ1 (Cernunnos/XLF)*, and *PRKDC (DNA-PKcs)* genes have been implicated in inborn immunodeficiencies (Table 13.2) (Buck et al., 2006; Corneo et al., 2000; Moshous et al., 2001; O'Driscoll et al., 2001; Schwarz et al., 1996; van der Burg et al., 2009; Villa et al., 1998).

Table 13.1 **MECHANISTIC STEPS IN V(D)J RECOMBINATION AND PROTEINS INVOLVED***

STEPS	PROTEINS
Lymphocytes-Specific Steps	
1. Locus accessibility	?
2. RSS recognition and nicking	RAG1 + RAG2
3. Synapsis of RSS	RAG1 + RAG2+?
4. Hairpin formation	RAG 1 + RAG2
General DNA Double-Strand Break Repair Steps	
5. Hairpin opening	Artemis and RAG1 + RAG2
6. Modification of coding ends	TdT + exonuclease(s)?
7. Recognition of DNA double strand. Disassembly of synaptic complex	DNA-PKcs + Artemis
8. Ligation	XRCC4 + Ligase IV+ Cernunnos

RSS, recombination signal sequence; TdT, terminal deoxynucleotidyl transferase.
*Alternative models suggest that steps 2 and 3 are interchangeable and that step 7 may precede steps 5 and 6.

COMPLETE *RAG1* AND *RAG2* | DEFICIENCY (B^- T^- SCID)

HISTORY

Siblings with diarrhea, candidiasis, lymphopenia, and diminished lymphoid tissue were first described by Glanzman and Riniker (1950). Thymic dysplasia and hypogammaglobulinemia were subsequently analyzed in new cases (Cottier, 1958; Hitzig et al., 1958). Knowledge of the antigen receptor gene structures and their processing allowed the definition of recombinase defective patients (Abe et al., 1994; Schwarz et al., 1991). A subgroup of patients with defective V(D)J recombination exhibited *RAG1/2* mutations (Schwarz et al., 1996).

DEFINITION

The functional failure of one of the constituents of the V(D) J recombinase machinery, such as RAG1 or RAG2, MIM (Mendelian Inheritance in Man) numbers 179615 and 179616, respectively, results in a SCID without B or T cells (B^- T^- SCID).

Table 13.2 CONSTITUENTS OF THE RECOMBINASE MACHINERY

	HUMAN GENE LOCUS	HUMAN DISEASE	CELL LINES
RAG1	11p13	SCID	AMuLV-transformed pre-B cells
RAG2	11p13	SCID	AMuLV-transformed pre-B cells
TdT	10q23–24	–	–
Ku80	2q33–35	–	XRCC5 cells (xrs5, 6, XR-V9B, XR-V15B, sxi-1, -2, -3, etc.)
Ku70	22q13	–	XRCC6
DNA-PKcs	8qll	RS-SCID	XRCC7
XRCC4	5q13	–	XRCC4
Ligase IV	13q33–34	Leaky SCID	Human fibroblast
Artemis	10p13	RS-SCID	Human fibroblast
Cernunnos	2q35	RS-SCID	Human fibroblast

AMuLV, Abelson murine leukemia virus; DNA-PKcs, DNA-protein kinase–dependent catalytic subunit; KO, artemis legend accordingly.

CLINICAL MANIFESTATION

Severe combined immunodeficiency (SCID) has an estimated incidence of approximately 1 per 100,000 live births (Stephan et al., 1993). In the original report, 6 of 30 SCID cases analyzed exhibited a RAG1 or RAG2 defect; thus the RAG deficiencies may account for a substantial proportion of human SCID cases (Schwarz et al., 1996). In a larger series, about 20 percent of SCID cases were RAG deficient (Villa et al., 2001; reviewed in Sobacchi et al., 2006). The clinical presentation is relatively uniform. As a rule, no symptoms are detected during pregnancy, at birth, or within the first few weeks of life. In most cases, the symptoms start within the second or third month after birth. Infectious complications are the hallmark of the disease, with a high preponderance of opportunistic infections (e.g., *Pneumocystis jiroveci* infection). The clinical presentation is characterized by chronic, persistent disease of the airways, recurrent acute pneumonia, therapy-resistant mucocutaneous candidiasis, eczematous dermatitis, and local as well as systemic bacterial infections (otitis, mastoiditis, purulent rhinitis and conjunctivitis, systemic sepsis, meningitis, arthritis, and localized abscesses). The recurrent infections as well as chronic enteritis lead to growth failure. Furthermore, intracellular pathogens (*Listeria*, *Legionella*) as well as viruses (Epstein-Barr virus [EBV] and cytomegalovirus [CMV]) may cause lethal complications.

Noninfectious clinical manifestations may result from graft-versus-host disease (GVHD). Because of the immunodeficiency, patients cannot reject allogeneic cells. Allogeneic cells can be introduced into patients either through maternofetal transfusion at the time of birth or by supportive transfusion therapy with nonirradiated blood products. While GVHD due to maternal lymphocytes is usually relatively mild with erythroderma, eosinophilia, enteritis, and hepatitis, GVHD following unirradiated transfusion is frequently lethal. Vaccination with living organisms such as application of the bacillus Calmette-Guérin (BCG) strain may cause disseminated disease with fatal consequences. All SCID children die within a few months if they are not provided with hematopoietic stem cells to restore functional T-cell immunity. Physical examination of completely RAG-deficient patients reveals unusual infections and a characteristic absence of lymphatic organs. In most cases cervical lymph nodes and tonsils are undetectable.

LABORATORY FINDINGS

Patients lacking RAG1 or RAG2 exhibit no B or T cells of their own in the peripheral blood ($B^- T^-$ SCID) (Table 13.3). Maternal T lymphocytes can be detectable in some cases and functional natural killer (NK) cells are present. After loss of the initially present maternal transplacentally transfused IgG, no antibodies circulate in the peripheral blood of RAG-deficient patients. In vivo and in vitro functional lymphocyte tests are abnormal because of the lack of the respective cells.

The RAG1 and RAG2 deficiencies are autosomal recessive diseases. Both genes are located on chromosome 11p 13 (Oettinger et al., 1992; Schwarz et al., 1994). Carriers of the mutant genes are healthy without any immunological disturbances and are therefore detected only through molecular identification of a mutation identified in the affected patient. A $B^- T^-$ SCID phenotype in umbilical cord blood may suggest, among other gene defects, a *RAG* mutatnt genotype that must be confirmed by molecular analysis.

RAG GENE STRUCTURE AND FUNCTION

The murine *Rag1* and *Rag2* genes were initially identified and cloned on the basis of their ability to rearrange an integrated artificial recombination substrate in a cell line (Oettinger et al., 1990; Schatz et al., 1989). The two complementing genes show a unique organization. Their 3' ends face each other and are

Table 13.3 **LABORATORY FINDINGS IN PERIPHERAL BLOOD OF RECOMBINATION-DEFICIENT PATIENTS**

	RAG-SCID	OMENN SYNDROME	ARTEMIS RS-SCID
B cells	–	–	–
T cells	–*	+ (oligoclonal)	–
NK cells	+	+	+
Immunoglobulin	–	– (or low)	–
Function **(in vivo and in vitro)**			
B cells	**NA**	**– very low**	**NA**
T cells	**NA**	**– (or low)**	**NA**
NK cells	+	+	+
Radiosensitivity	–	–	+

NA, not applicable; NK, natural killer. *After exclusion of maternally transfused T cells, which can be detected in more than 50% of the cases.

separated in human DNA by 15–18 kb. The coding sequences and the 3' untranslated region (UTR) of each gene is located in a single exon (Ichihara et al., 1992). *RAG1* possesses one extra 5' UTR exon, whereas in the *RAG2* locus at least two 5' UTR exons have been identified (Lauring and Schlissel, 1999). The amino acid sequence of both RAG genes and the overall genomic organization are highly conserved throughout evolution from sea urchins to humans (Bernstein et al., 1994; Fugmann et al., 2006; Wienholds et al., 2002). The human *RAG1* gene encodes 1403 amino acids; the *RAG2* gene, 527 amino acids.

RAG1 and RAG2 protein sequences are not related to each other. A sequence comparison of RAG1 with other proteins shows that RAG1 possesses five basic regions that are necessary for nuclear localization (binding sites for nuclear protein SRP1 and RCH1), a region with homology to bacterial invertases and homeodomain proteins, zinc finger domains, and a zinc binding dimerization motif (Plate 13.III) (Rodgers et al., 1996; Silver et al., 1993; Spanopoulou et al., 1996). Extensive mutagenesis of acidic amino acids in RAG1 identified three catalytic residues (D600, D708, and E962, the so-called DDE motif) critical for both nicking and hairpin formation (Landree et al., 1999); two of these (D600 and D708) coordinate catalytic divalent metal ions (Fugmann et al., 2000b; Kim et al., 1999; Landree et al., 1999). These residues are located in a region that displays marked conservation in predicted secondary structure with the catalytic cores of other transposases (Fugmann et al., 2000b). Further insight into RAG1 activities has been derived from mutants blocking the hairpin formation, which are all in the vicinity of D600 (Kale et al., 2001). Two regions of RAG1, the nonamer binding domain and the carboxyterminal domain, contact DNA containing the coding flank at the cleavage site (Mo et al., 2001). Recently, a RAG1 nonamer binding domain (NBD) has been demonstrated to form a tightly interwoven dimer that binds and synapses two nonamer elements, with each NBD making contact with both DNA molecules. Biochemical and biophysical experiments have confirmed that the two nonamers are in close proximity in the RAG1/2-DNA synaptic complex (Yin et al., 2009).

The molecular roles of RAG2 in V(D)J recombination are less known. The presence of RAG2 is required for all catalytic steps and helps to form the RAGRSS complex. Secondary structure prediction and mutagenesis studies have suggested that RAG2 adopts a six-bladed β-propeller fold (Fig. 13.1) (Callebaut and Mornon, 1998; Corneo et al., 2000; Gomez et al., 2000), a structural motif found in many proteins of

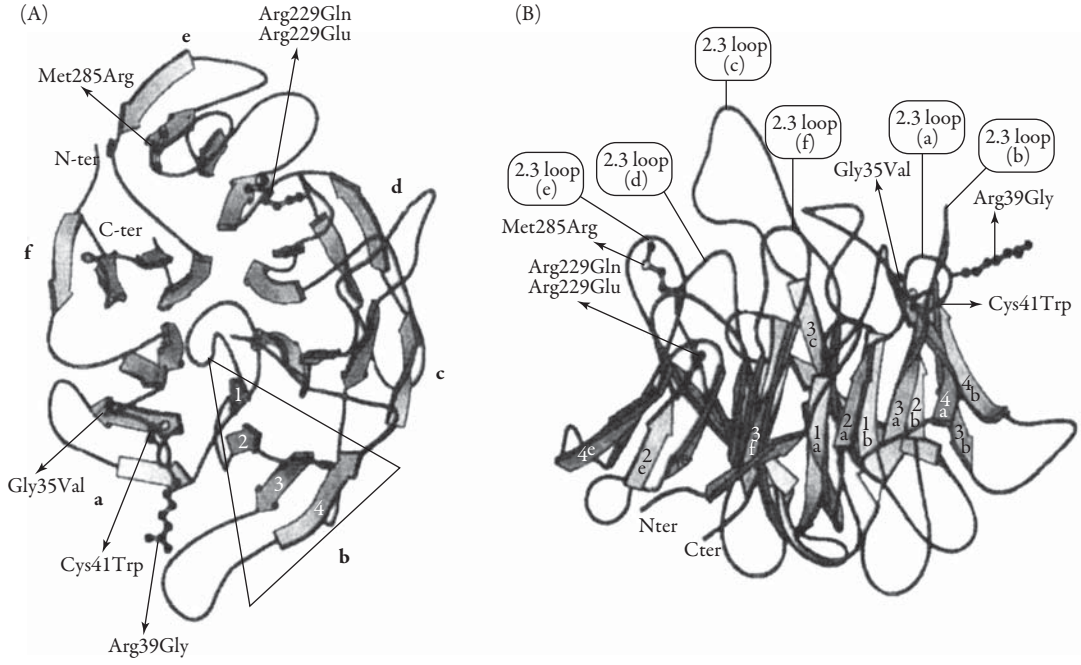

Figure 13.1 (A) Beta-propeller structure of RAG2. Each blade of the propeller consists of a four-stranded beta sheet. (B) Beta-propeller structure viewed along the perpendicular axis. Mutated amino acids are indicated.

diverse function (Adams et al., 2000). The core region of RAG2 (aa 1–382 out of 527) assists RAG1 interaction with the RSS and is essential for DNA distortion. The C terminus of RAG2 includes a hinge domain with a high percentage of acidic residues that connects the core region to a noncanonical plant homeo domain (PHD) finger (Callebaut and Mornon, 1998; Corneo et al., 2002; Elkin et al., 2005), which overlaps a phosphorylation site at residue T490 implicated in the cell-cycle-regulated degradation of RAG2 (Li et al., 1996; Lee and Desiderio, 1999; Lin and Desiderio, 1993; Ross et al., 2003). Furthermore, this region has been implicated in binding to the core histone proteins (West et al., 2005). Importantly, in B cells the C-terminal region of RAG2 is important for IgH V to DJ rearrangement (Kirch et al., 1998).

Another role of RAG proteins is their ability to catalyze in vitro transpositional insertion, which suggests that this process could be a source of genomic instability in vivo (Agrawal et al., 1998; Hiom et al., 1998). Indeed, RAG transposase forms productive complexes with target DNA both before and after RSS cleavage, and these show a preference for transposition into nearby targets, such as Ig and TCR loci. This could bias transposition toward relatively safe regions in the genome (Neiditch et al., 2001). In addition, the transposition events are stimulated and targeted by the presence of distorted DNA structures such as hairpins. Because there is no evidence that RAG-mediated transposition occurs in vivo, it is likely that regulatory mechanisms limit the frequency of transposition events in lymphocytes. During lymphocyte development in adults, the *RAG* genes are convergently transcribed in thymic and bone marrow cells with a capability for V(D)J recombination (Oettinger et al., 1990; Schatz et al., 1989). Mature RAG proteins are localized to the nucleus in developing thymocytes. RAG expression is first detected in committed double-negative (DN) thymic T-cell precursors, whereas the first B cells expressing RAG1 and RAG2, at very low levels, are AA 4.1^+ HAS^- $B220^+$ $CD4^+$ $CD43^+$. RAG expression increases as B cells mature. Following the proliferative expansion of pre-B and pre-T cells, a second wave of RAG expression is initiated in $CD25^+$ pre-BII B lineage cells and $CD4^+$ and $CD8^+$ double-positive (DP) early T cells. This second expression has been shown to be regulated by elements at the 5' end of RAG2 promoter (Yu et al., 1999). RAGs are not expressed in mature T cells. Immunohistochemical analysis detected RAG expression in germinal centers in the mouse (Han et al., 1996; Hikida et al., 1996); however, transgenic indicator and gene-targeted indicator lines proved that the small number of cells expressing RAG are immature B cells (reviewed in Nagaoka et al., 2000).

It has been suggested that a RAG1/RAG2 complex nucleates a specialized subnuclear compartment named the V(D)J recombination factory (Matthews and Oettinger, 2009). This molecular machine does more than merely catalyze the phosphoryl-transfer reactions during DNA DSB generation. In addition to cutting DNA, the RAG1/RAG2 complex interacts with methylated histones (H3K4me3), stimulating the complex to perform both nicking and hairpinning in *trans* in addition to tethering in *cis* (Shimazaki et al. 2009). The complex is involved in immunoglobulin allelic pairing and helps determine whether the V(D)J recombination DNA breaks will be repaired by classical NHEJ (cNHEJ), altenative NHEJ (aNHEJ), or homologous recombination.

MUTATION ANALYSIS

Mutagenesis experiments have defined core regions for RAG1 and RAG2 that are necessary and sufficient to recombine extrachromosomal V(D)J recombination substrates. The RAG1 core includes amino acids 392–1011, whereas the RAG2 core extends from amino acids 1–382 (Cuomo and Oettinger, 1994; Sadofsky et al., 1993, 1994; Silver et al., 1993). After the first description of T^- B^- SCID cases with RAG defects, many additional mutations have been detected in RAG1 and RAG2 (Corneo et al., 2000, 2001; Gomez et al., 2000; Sobacchi et al., 2006; Villa et al., 2001). Mutation analysis of RAG1 and RAG2 (http://bioinf.uta.fi/RAG1base and http://bioinf.uta.fi/RAG2base) indicates that most are null mutations (nonsense or frameshift). In addition, although some patients with classical T^- B^- SCID bear missense mutations, biochemical studies have shown that these amino acid changes completely abrogate recombination and therefore represent functionally null alleles (Corneo et al., 2000; Gomez et al., 2000; Schwarz et al., 1996). In transient transfection assays with artificial extrachromosomal rearrangement substrates, the mutants derived from completely RAG-deficient SCID patients show only residual recombination values that are typically 0.1 to 1 percent of wild type for both coding and signal joint formation. Pre-B and pre-T cells do not survive during lymphocyte development if they do not obtain a survival signal from their respective pre-B and pre-T cell receptors. The heavy chain of the IgM molecule and the TCR chain are necessary constituents of the respective receptors. In complete RAG deficiency, V(D)J recombination cannot be initiated; thus IgM and TCR chains are not synthesized and pre-B or pre-T cell receptors are not expressed. Such precursor lymphocytes receive no survival signals and consequently are eliminated, hence the alymphocytosis in patients with complete RAG1 and RAG2 deficiency.

DIAGNOSIS

At present, no simple functional test exists to reveal a V(D)J recombinase defect in general or a specific RAG defect. Protein analysis is not a practical alternative because the recombining cells are a minority within the bone marrow or require a thymic biopsy. Thus, direct sequencing of *RAG* gene DNA is essential, with functional assessment of effect on recombination by in vitro analysis if necessary.

TREATMENT

Treatment of RAG-defective children consists of a combination of supportive measures (see Chapter 59) and curative therapy by hematopoietic stem cell transplantation (HSCT) (see Chapter 60). An HLA-identical donor is no longer a prerequisite for reconstitutive HSCT, since depletion of T cells

from a nonidentical graft omits or greatly reduces the risk of GVHD. When indicated, patients may be transplanted prior to conditioning because their immunodeficiency makes graft rejection unlikely. Survival after transplantation in RAG-deficient SCID is historically around 65 percent. In utero bone marrow transplantation (BMT) has been performed in selected patients (Pirovano et al., 2004). An alternative therapeutic approach could be gene therapy (Cavazzano-Calvo et al., 2000; Fischer et al., 2002; Lagresle-Peyrou et al., 2008). Although there have been no studies of *RAG2* gene therapy in humans, experiments of retrovirally mediated *Rag2* gene transfer in hematopoietic stem cells of *Rag2* knockout mice showed good results (Yates et al., 2002). Recently, Wagemaker et al. developed new third-generation lentivital vectors carrying a *Rag2* gene able to overcome the T- and B-cell block in the *Rag2* knockout mouse, thus opening the door to clinical application (N. van-Til, personal communication).

ANIMAL MODELS OF RAG1/2 DEFICIENCY

Mice with homozygous deletions of *Rag1* or *Rag2* have been created by gene targeting (Mombaerts et al., 1992; Shinkai et al., 1992). Both exhibit an identical phenotype to that of each other and of human RAG-deficient patients. The animals cannot initiate V(D)J recombination, and thus lack B and T cells. B-cell development is halted at the pre-B cell stage (Diamond et al., 1997; Mombaerts et al., 1992; Shinkai et al., 1992). Functional defects in Rag-deficient mice appear limited to the immune system. One report stated that Rag1 knockout mice had increased locomotor activity and reduced levels of fearfulness (Cushman et al., 2003).

Rag2 mice generated by replacing the endogenous Rag2 locus with core Rag2 lacking the "dispensable" C-terminal domain had reduced B- and T-cell numbers (Akamatsu et al., 2003; Liang et al., 2002). This finding demonstrated that the C-terminal domain, which is dispensable for V(D)J recombination in vitro, nonetheless has relevant functions in vivo.

PARTIAL *RAG1* AND *RAG2* DEFICIENCY (OMENN SYNDROME)

Omenn syndrome (OS) (MIM 267700) is a rare disorder that was long an enigma for pediatricians and immunologists (Omenn, 1965). OS is a rare autosomal recessive disease characterized by symptoms of SCID plus erythroderma, eosinophilia, hepatosplenomegaly, lymphadenopathy, and elevated serum IgE levels, suggesting a defect in the activation and/or regulation of T-cell proliferation. The identification of specific mutations of *RAG* genes in OS patients made it clear that the underlying defect affects the maturation of both T and B lymphocytes and that T cells are activated secondary to a partially defective V(D)J recombination process (Villa et al., 1998). However, the occurrence of the same RAG gene mutations in patients with T^- B^- SCID as with OS raises the possibility that additional factors such as epigenetic factors influence development of the Omenn phenotype (Corneo et al., 2001; Villa et al., 2001).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Patients with OS have early-onset generalized erythroderma, lymphoadenopathy, hepatosplenomegaly, fever, protracted diarrhea, and failure to thrive (Plate 13.IV, Table 13.4). Protein loss due to diarrhea and exudative erythroderma often leads to generalized edema. The presence of a massive inflammatory infiltrate gives the skin a unique appearance and consistency (pachydermia). Alopecia is frequent (Aleman et al., 2001). Despite the presence of lymph nodes and variable, often elevated, numbers of circulating T lymphocytes, OS patients are highly susceptible to bacterial, viral, and fungal infections. Unless treated by HSCT the disease is invariably fatal. Infections and malnutrition are the main causes of death (Gomez et al., 1995). Septicemia, often arising from skin infections, is common. The clinical hallmarks of the disease are reminiscent of GVHD. Indeed, in some cases the occurrence of maternal T-cell engraftment or unirradiated blood-product transfusion in infants with SCID may result in clinically overt GVHD and mimic OS (Anderson and Weinstein, 1990; Le Deist et al., 1987; Pollack et al., 1982). For a diagnosis of OS, it is therefore essential that, in addition to typical clinical and laboratory findings, allogeneic T-cell engraftment be excluded. The similarity of OS to GVHD is further reinforced by the pathological features of OS. Skin biopsies reveal lymphocytic infiltrates in the upper dermis, with occasional histiocytes and eosinophils (Dyke et al., 1991). Immunohistochemical analysis shows that the lymphocytic infiltrate is composed of activated ($CD45RO$, DR^+) T cells, many of which co-express CD30, a surface molecule associated with Th2-type cytokine secretion (Chilosi et al., 1996b). The differential diagnosis of OS includes severe atopic dermatitis, GVHD, and histiocytosis X, known as Letterer Siwe syndrome (Aleman et al., 2001). Lymph node enlargement, typically observed in OS, is in contrast to the paucity of peripheral lymphoid tissue usually detected in patients with combined immune deficiencies. However, the lymph node architecture is severely altered in OS, with lack of follicles, depletion of the normal lymphocytic population, and increased proportion of interdigitating reticulum cells and eosinophils (Barth et al., 1972; Dyke et al., 1991; Martin et al., 1995; Omenn, 1965). On the basis of these findings, the disease was originally also named *familial reticuloendotheliosis with eosinophilia* (Omenn, 1965) or *combined immunodeficiency and reticuloendotheliosis with eosinophilia* (Ochs et al., 1974). Immunohistochemistry indicates that the lymphocytic component in the lymph nodes is similar to that in skin infiltrates (Brugnoni et al., 1997; Chilosi et al., 1996a, 1996b). Interestingly, staining with BlimpI and CD138 reveals the presence of positive immunoglobulin secreting cells (ISCs) in the lymph nodes of the patients (Cassani et al., 2010). The splenic white pulp, Peyer's patches, and lamina propria of the gut are also markedly depleted of lymphocytes. The thymus, which is profoundly hypoplastic, has a noticeable depletion of lymphoid components and often lacks Hassall's bodies (Barth et al., 1972; Businco et al., 1987). Indeed thymic biopsies obtained from OS patients have allowed the evaluation of the thymic architecture and cellular distribution, with particular

attention to the presence of dendritic cells (DCs) and natural regulatory T cells (nTreg). Analysis of medullary thymic epithelial cells showed in the OS thymus absence of Aire expression, a transcription factor driving the expression of tissue restricted antigens that are presenting to autoreactive T cells, inducing deletional central tolerance (Cavadini et al., 2005). Furthermore, immunohistochemical analysis with S100, CD11c, and Foxp3, staining DCs and nTreg respectively, showed virtual absence of thymic DCs and lack of Foxp3+ cells (Poliani et al., 2009). All these findings, the lack of Aire expression, the severe depletion of thymic Foxp3+ cells may provide a mechanism to explain the pathophysiology of autoimmune manifestations in Omenn syndrome.

LABORATORY FINDINGS

As summarized in Table 13.4, the main laboratory findings in OS consist of eosinophilia, hypogammaglobulinemia with increased serum IgE, presence of activated circulating T lymphocytes, in contrast to the usual lack of peripheral blood B cells, and a poor in vitro proliferative response of T lymphocytes to specific antigens (with variable responses to mitogens). Additional laboratory findings include anemia (and sometimes thrombocytopenia) and hypoproteinemia due to protein loss through the stools and the skin. In contrast to most forms of combined immunodeficiency, the total number of circulating T cells is variable but often elevated (Table 13.3). Distribution of the main T-cell subsets (i.e., CD4 vs. CD8) is frequently imbalanced (Brugnoni et al., 1997; Businco et al., 1987; Karol et al., 1983; Le Deist et al., 1985). An extreme example of the variability in T-cell number in OS (which offered a crucial clue to the molecular basis of the disease) is represented by a pedigree in which one patient had typical OS with increased circulating T cells, whereas one of his younger brothers died with T^- B^- SCID (de Saint Basile et al., 1991).

Table 13.4 **DIAGNOSIS OF OMENN SYNDROME: CLINICAL AND LABORATORY HALLMARKS**

Clinical Features
Early-onset, generalized erythroderma
Failure to thrive
Protracted diarrhea
Edema
Lymphadenopathy
Hepatosplenomegaly
Severe infections (pneumonia, sepsis)
Laboratory Features
Hypoproteinemia
Frequent anemia, thrombocytopenia
Remarkable eosinophilia (usually >1,000/μL)
Very low IgG, IgA, and IgM, but usually increased IgE
Very low or absent circulating B cells
Variable (often elevated) number of activated ($CD45R0^+$, DR^+) circulating T cells
Very low in vitro proliferative responses to antigens, variable response to PHA
Low, IgM-restricted, antibody responses

Many of the immunological hallmarks of OS reflect the presence of activated T cells skewed to a Th2 phenotype. Similar to skin and lymph nodes, circulating T cells express activation markers (CD45R0, DR, CD25, CD95, CD30) and secrete Th2-type cytokines (IL-4, IL-5) upon in vitro activation. Accordingly, serum levels of IL-4 and IL-5 are increased. In contrast, serum levels and in vitro production of IL-2 and IFN-γ are reduced (Brugnoni et al., 1997; Chilosi et al., 1996a; Schandene et al., 1993). The IL-4 and IL-5 may drive increased production of IgE and eosinophilia, respectively. Finally, invariant natural killer T (iNKT) cells, important immunoregulatory cells, are absent in OS patients (Matangkasombut et al., 2008).

Although serum IgG, IgA, and IgM are markedly reduced in OS, specific antibody production is not completely impaired (Le Deist et al., 1985), indicated by a measurable although low and IgM-restricted response to immunization with bacteriophage x174 (Ochs et al., 1974). Hypogammaglobulinemia is partially due to protein loss but also reflects defective B-cell differentiation, with a very low or absent number of circulating B cells. Because B lymphocytes are usually undetectable in the lymph nodes and the gut, it remains unknown where the IgE secretion occurs. Although T lymphocytes are consistently present and show an activated phenotype, they are functionally defective, with reduced proliferative responses to antigens and occasionally to mitogens as well (Brugnoni et al., 1997; Businco et al., 1987; Harville et al., 1997; Le Deist et al., 1985). Oligoclonality of circulating T cells has been consistently described in OS (de Saint Basile et al., 1991; Harville et al., 1997; Rieux-Laucat et al., 1998; Villa et al., 1998; Wirt et al., 1989). Although the use of distinct variable (V) gene segments is not particularly biased, few clones are expanded within each population of T cells that express specific V genes, as demonstrated by sequence analysis showing sets of identical V(D)J sequences (Harville et al., 1997; Villa et al., 1998). It has been hypothesized that restriction of the TCR repertoire could arise in the periphery or in the thymus. Analysis of T cells from both thymus and peripheral blood from a deceased OS patient demonstrated that the TCRβ repertoire was already restricted in the thymus, although further selection occurred in the periphery (Pirovano et al., 2003; Signorini et al., 1999). Defects in regulatory T cells leading to a breakdown of peripheral tolerance have been described, along with a defect in central tolerance explaining the occurrence of autoimmune manifestations (Cassani et al., 2010a; Somech et al., 2009).

MUTATION ANALYSIS IN PATIENTS WITH OMENN SYNDROME

The rarity of OS made positional cloning of a responsible gene impossible, and as mentioned above, the unique clinical picture of OS gave few reasons to suspect that *RAG* mutations could be the cause. However, one hint came from descriptions

of the occurrence of OS and SCID within members of the same family (de Saint Basile et al., 1991). Gene identification was also complicated by the similarities between OS and SCID with GVHD. Nevertheless, the analysis of *RAG* genes showed mutations in OS patients (Corneo et al., 2001; Villa et al., 1998). To explain why a defect in the same genes can give rise to such different phenotypes, factors such as genetic background, modifier genes, and epigenetic events were evoked. However, it is now clear that mutations in different domains of the *RAG* genes can affect the protein structure to various extents, leading either to null alleles or to hypomorphic alleles retaining variable degrees of function. Careful analysis supports the hypothesis that while SCID represents "null" RAG alleles, the OS phenotype is caused by RAG alleles that retain partial V(D)J recombination activity. According to this view, OS is a "leaky" SCID, allowing some degree of maturation along the T-cell lineage to occur. In contrast to the mutations noted in T^- B^- SCID patients, OS patients show predominantly missense mutations, with at least one missense mutation present in every patient. This finding, together with the presence of oligoclonal cells in the periphery, led us to suggest that limited V(D)J recombination events occur in these patients. Biochemical assays analyzing the capacity to mediate full V(D)J recombination events, *SCC* formation, and the introduction of DSBs revealed that the proteins containing missense mutations had reduced but still detectable activity. Hence, in general, SCID patients have two entirely defective alleles, whereas OS patients have at least one allele that is partially functional and capable of establishing the restricted receptor repertoire seen in OS (Corneo et al., 2001; Kumaki et al., 2001; Noordzij et al., 2002; Villa et al., 1998, 2001; Wada et al., 2000).

Particularly interesting is the description of OS cases with alleles carrying nucleotide deletions in the N-terminal domain. In fact, an N-terminus-truncated active RAG1 protein is generated from these alleles by reinitiation from internal in-frame methionine residues downstream of the deletion (Noordzij et al., 2000; Santagata et al., 2000). The biochemical analysis of both null and hypomorphic mutations in *RAG1* and *RAG2* have provided valuable insights into the biology of the RAG1/RAG2 recombinase. The importance of the NBD has been reinforced by the finding that over 30 percent of *RAG1* mutations in OS patients result in amino acid substitutions in the NBD, a region spanning only 4 percent of the molecule.

The structural model of Rag2 as a six-bladed β-propeller with a C-terminal PHD finger has been supported by functional data from a number of identified OS and SCID mutations. Amino acid substitutions in three SCID patients were localized to the second β strand of the first, second, and fourth kelch repeats of RAG2 and were shown to abrogate the V(D)J recombination activity of the altered proteins (Corneo et al., 2000; Gomez et al., 2000). Moreover, many of the null and hypomorphic RAG2 active core mutations have a spatial clustering on one face of the predicted β-propeller, thereby defining a potentially critical surface for interaction with RAG1 (Corneo et al., 2000). Further support of the structural model of RAG2 has been provided by the identification of four mutations leading to amino acid substitutions in the C-terminal PHD finger (C423Y, W453R, N474S, and C478Y). The W453R substitution was identified in an OS patient and has residual levels of recombination activity (Gomez et al., 2000), while the other three substitutions were found in T^- B^- SCID patients and are likely inactive for recombination. Two of the altered amino acids, C423 and C478 (Schwarz et al., 1996, A. Villa et al., unpublished results), are predicted to coordinate zinc ion and are therefore entirely conserved in all known PHD fingers.

Finally, the importance of hypomorphic RAG mutations in sustaining the OS phenotype is also demonstrated by the case of an infant who presented with T^- B^- SCID because of homozygosity for a null RAG1 mutation and then developed a typical OS phenotype because of somatic mosaicism with multiple second-site mutations that restored RAG1 expression (Wada et al., 2005).

MUTATION ANALYSIS IN PATIENTS WITH ATYPICAL AND LEAKY SCID

The analysis of a large series of immunodeficient cases with RAG defects has permitted the identification of a new group of patients with some, but not all, of the clinical and immunological features of OS, a condition we call "atypical SCID/OS." In molecular terms this category appears more like OS than classic SCID because all of patients carry at least one missense mutation, some of which are also found in OS patients. This finding supports the idea that in these atypical patients, partial RAG activity is responsible for the development of a low number of T and possibly B lymphocytes. A third phenotype, characterized by mutations in RAG1, CMV infection, and oligoclonal expansion of TCRγδ T cells, has been described by two independent groups (de Villartay et al., 2005; Ehl et al., 2005), demonstrating that environmental factors can add to the complexity of the clinical and immunological phenotype.

An additional phenotype of hypomorphic RAG combined immunodeficiency (CID) was characterized by granulomas in the skin, mucous membranes, and internal organs. All patients had severe complications after viral infections, including B-cell lymphoma associated with EBV. Other findings were hypogammaglobulinemia, a diminished number of T and B cells, and sparse thymic tissue on ultrasonography. Moreover, the patients were diagnosed as children (3 to 11 years), demonstrating that "classical" SCID genes must be considered in the diagnostic workup of childhood presentation of CID (Schuetz et al., 2008).

Overall, these findings indicate that partial RAG activity may be a prerequisite for OS or CID, but other epigenetic factors are needed to understand the disease (Corneo et al., 2001; Villa et al., 2001). Alternatively, it is possible that individual differences such as early or delayed medical treatment could contribute to clinical and immunological heterogeneity, particularly because some patients undergo HSCT very early in the course of the disease and this treatment could prevent the development of typical OS findings.

However, not all cases of OS are due to defects in RAG genes. OS was also identified in one patient with mutations in the DLCRE1C gene, encoding Artemis, a factor mediating hairpin coding end opening during the V(D)J recombination process (Ege et al., 2005). The patient was a compound heterozygote; 1 allele carried a hypomorphic mutation (M1T) that preserved residual V(D)J activity (2.1 to 2.7 percent). Moreover, mutations in DNA-ligase IV were reported to cause features of OS (Grunebaum et al., 2008). Interestingly mutations in this gene have been previously found in a variety of clinical settings, including leukemia, lymphoma, and bone marrow failure, and in a subset of patients with absent T and B cells (Enders et al., 2006, van der Burg et al., 2006).

Beyond the involvement of VDJ recombination factors in the pathogenesis of OS, other gene defects have been described to cause OS. Indeed, mutations in *RMRP*, associated with cartilage-hair hypoplasia (CHH) (see Chapter 37), have been reported in two patients presenting with clinical features consistent with OS (Roifman et al., 2006). Two unrelated cases of adenosine deaminase deficiency had clinical features of OS (Roifman et al., 2008). Finally, OS with hypomorphic defects in the common γ-chain, the IL-7 receptor α chain, and the chromodomain helicase DNA binding protein 7 (CHD7) have been reported, supporting the concept that OS represents an aberrant inflammatory condition brought about by different genetic defects (Marrella et al., 2011; Villa et al., 2008).

TREATMENT

Unless treated by HSCT, OS is invariably fatal because of infections and/or malnutrition. The clinical presentation is usually so severe that supportive treatment is warranted even before a diagnosis is formally established.

Despite this broad supportive treatment, the clinical status of OS patients waiting for HSCT often remains critical, mainly because of cutaneous and intestinal problems directly related to deranged T-cell activation. In an attempt to overcome the activation of Th2 cells and to achieve better control of disease activity, different strategies have been used. The daily use of IFNγ (the rationale being to restore the balance between Th1- and Th2-type cytokines) succeeded in ameliorating clinical conditions in one infant, concurrently with a decrease in eosinophil count and an increase in lymphocyte proliferation to mitogens (Schandene et al., 1993). Other groups have tried to block T-cell activation using immunosuppressive drugs. Steroids have proven ineffective or only partially effective (Barth et al., 1972; Le Deist et al., 1985; Ochs et al., 1974; Omenn, 1965). Somewhat better results have been obtained with cyclosporine A (Brugnoni et al., 1997; Wirt et al., 1989). However, the potential beneficial effect of immunosuppressive drugs and IFNγ is of limited duration.

Because of the specific features of OS, the overall results of HSCT in these children are less satisfactory than in other forms of combined immunodeficiency. The first attempts reported were disappointing, as only 7 of 26 patients were cured (Barth et al., 1972; Bruckmann et al., 1991; Fischer et al., 1990, 1994; Heyderman et al., 1991; Junker et al., 1988; Loechelt et al., 1995; Schofer et al., 1991). A high frequency of graft failure was observed. Aggressive conditioning with myeloablative and immune-suppressive drugs is needed to circumvent this problem. More recently, the use of appropriate supportive treatment and prophylaxis of infection, together with tailored conditioning regimens, has resulted in better outcomes. Taking advantage of these advances, Gomez et al. (1995) reported the cure of OS by HSCT in six of nine patients; similarly, another group achieved successful treatment in four of five OS patients (Brugnoni et al., 1997; Chilosi et al., 1996a). The successful engraftment of donor-derived hematopoietic stem cells is associated with the development of normal numbers and functions of both T and B lymphocytes, with full clinical and immunological recovery.

ANIMAL MODELS OF RAG DEFECTS

Although the molecular bases of OS have been extensively analyzed, several aspects of the disease have not yet been clarified. Selective expansion of certain TCR clones may be a consequence of either intrathymic selection of specific rearrangements or peripheral expansion in response to infections or perhaps autoantigens. It has been suggested that given the extreme disorganization of the thymic microenvironment in OS, negative selection of autoreactive clones may be inoperative, and a few residual T-cell clones may expand in the periphery because of their autoreactive character (Fischer and Malissen, 1998; Marrella et al. 2007, 2008). Another unexplained phenomenon is the fact that RAG proteins direct recombination in both B and T cells, yet B cells are absent in the peripheral blood of OS patients. In view of the elevated IgE levels in OS patients, it is clear that somewhere, B cells that perform Ig rearrangements and switch to IgE production do exist. It is noteworthy that V(D)J recombination deficiencies that lead to low to normal numbers of T cells in the absence of mature B cells is a recurring theme. Another point that must be clarified is the role of environmental factors in the pathogenesis of the Th2 expansion and the resulting clinical features. As observed in other cases, immunodeficient individuals have by definition a more intense antigen exposure and a defect in antigen clearance that results in a persistent high antigen load. If, as in OS, the genetic defect is permissive and allows the development of limited clones of mature T cells, the antigen overload favors prolonged T-cell activation that has been associated with increased IL-4 secretion and polarization toward a Th2 phenotype (Hosken et al., 1995; Hsieh et al., 1993). The availability of murine models carrying the same mutations found in OS and leaky SCID patients helps to clarify the cellular and molecular mechanisms underlying these enigmatic aspects. To this aim, three independent groups have investigated the effects of three hypomorphic mutations of OS patients (Rag2 R229Q, Rag1 R972Q, and Rag1 S723C).

Mice with R229Q in Rag2, *rag2*$^{R229Q/\ R229Q}$, showed a peculiar phenotype, with 60 percent developing alopecia, skin erythroderma, wasting syndrome, and colitis (Marrella et al., 2007). Importantly, the skin and gut of the affected

animals had autoreactive T-cell infiltration with eosinophils. Hematopoietic organs showed severe alterations. The thymus had a reduced number of single-positive (SP) T cells in spite of the absence of DP thymocytes and a severe block at the DN3 stage ($CD44^{-}$ $CD25^{+}$) at which the TCRβ locus rearranges. Notably, few DP thymocytes showed upregulation of TCRα or β or of CD69, suggesting that positive selection was intact. Positively selected SP cells reaching the periphery displayed an oligoclonal repertoire and effector or memory-like phenotype $CD44^{+}$ $CD62L^{low}$ and were unresponsive to anti-CD3 mAb in vitro. As in OS patients, T cells showed an oligoclonal repertoire and circulating B cells were reduced.

There was profound hypogammaglobulinema in spite of high levels of IgE. B-cell differentiation in the bone marrow was arrested at the pre-B cell stage, lymph nodes lacked germinal centers, and no marginal-zone B cells were detected in the spleen. Despite the severe pre-B cell arrest, a few IgM^{+} B cells expressed activation markers and generated ISCs. In a scenario of severe lymphopenia, a compensatory homeostatic regulation and an increased transcriptional program led to the maturation of a few autoreactive B cells that expanded in the periphery, causing organ infiltration and damage (Cassani et al., 2010b). Importantly, *rag2*$^{R229Q/\ R229Q}$ mice had autoimmune manifestations attributed to defects in both central and peripheral tolerance. Indeed, analysis of Aire expression, a key factor controlling the negative selection process, was severely abrogated in the thymus, similarly to that observed in patients (Cavadini et al., 2005). Moreover, nTreg cells ($CD4^{+}$ $CD25^{high}$ $Foxp3^{+}$) were markedly reduced in all hematopoietic organs. Furthermore, as in OS patients, invariant natural killer (iNKT) cells, a population having a role in regulating adaptive and innate immune response, were completely absent.

In parallel to this model, an independent group described a spontaneous mutant mouse carrying a point mutation in Rag1 corresponding to the human amino acid R975 and showing a high percentage of memory phenotype T cells (Khiong et al., 2007). In these mice, named MM (memory mouse), T-cell development in the thymus was partially blocked at the DN3 stage, while spleens and lymph nodes showed elevated numbers of activated memory T cells ($CD69^{+}$, $CD44^{hi}$) and biased usage of TCRVβ and α, although less profound than the extremely severe oligoclonality in OS patients. In contrast to OS features but similar to the atypical form of SCID, MM mice displayed a bone marrow B-cell developmental arrest at the pro-B cell stage ($CD43^{+}$ $B220^{med}$), while splenic B cells arrested at the pre-B stage. B cells had a restricted spectratype suggesting an oligoclonal repertoire. In contrast with what was observed in patients, MM mice had higher serum immunoglobulins and no T-cell infiltration in tissues or autoimmunity in spite of T-cell activation marker expression and high production of Th2 cytokines IL-4 and IL-6. To explain abnormal cytokine production from $CD4^{+}$ T cells including Th2 subset, the authors demonstrated involvement of homeostatic proliferation of $CD4^{+}$ T cells, known as a trigger of autoimmune manifestations (King et al., 2004; Sawa et al., 2006). Thus, MM mice had some OS manifestations but more closely recapitulated the atypical forms of SCID.

Finally, a third mouse mutant, carrying an hypomorphic mutation S723C in Rag1, had few mature CD4 and CD8 SP αβ T cells in the peripheral lymphoid organs (Giblin et al., 2009). B cells were arrested at the pro-B stage. Furthermore, heterozygous mice developed age-associated immune dysfunction in T and B cells compared with age-matched controls. And interestingly, when backcrossed into a p53 mutant background, they developed thymic lymphomas associated with chromosomal translocations. The occurrence of cancer in *rag1*$^{S723C/7S723}$ mice in a $p53^{-/-}$ background revealed a possible implication of Rag hypomorphic mutations in the pathogenesis of tumors and premature immunosenescence.

In conclusion, the observations derived from these mouse models, showing alteration in homeostatic proliferation, failure in central and peripheral tolerance, allow us to better understand some of the clinical features shown in OS and atypical and leaky SCID.

RADIOSENSITIVE $T^{-}B^{-}$ SCID

DEFINITION

Some $T^{-}B^{-}$SCID patients lack mutation in either *RAG1* or *RAG2*, despite having the same clinical presentation (Table 13.3). The alymphocytosis in these patients is accompanied by an increased cellular sensitivity to ionizing radiation, or radiosensitivity (RS-SCID, MIM 602450 and 605988), a situation reminiscent of the well-known *scid* mouse, leading to the hypothesis of a general defect in the DNA repair machinery. The RS-SCID phenotype is also found with high incidence among Athabascan-speaking Native American Indians (1 in 2,000 live births among Navajo Indians) (Hu et al., 1988).

LABORATORY FINDINGS

Bone marrow cells (colony forming units-granulo-monocytes [CFU-GM]) and primary skin fibroblasts of patients with RS-SCID have increased sensitivity to ionizing radiation (Cavazzana-Calvo et al., 1993), as well as a defect in V(D)J recombination in fibroblasts in vitro (Nicolas et al., 1998). V(D)J recombination analysis in fibroblasts from Athabascan SCID patients suggested a common molecular defect (Moshous et al., 2000) and, therefore, the existence of a new V(D)J recombination factor. The disease-related locus was assigned to the short arm of human chromosome 10 by linkage analysis (Li et al., 1998; Moshous et al., 2000).

THE ARTEMIS FACTOR

Given the location of the RS-SCID gene on human chromosome 10, genomic DNA sequences released were systematically analyzed using two computer programs, FGENESH and GENESCAN, aimed at identifying putative genes. On the basis of a putative peptide proposed by these programs, a full-length cDNA coding for a new factor called *Artemis* was isolated (Moshous et al., 2001). Functional complementation and

mutation analyses certified that Artemis was indeed defective in RS-SCID. As expected, because of the ubiquitous increase of cellular radiosensitivity in RS-SCIDs, Artemis was expressed in every tissue tested. Although Artemis does not have any global homologs in the databases, BLAST search analyses revealed significant similarities of the first 150 amino acids to members of the metallo-β-lactamase superfamily. The metallo-β-lactamase fold, first described for the *Bacillus cereus* β-lactamase (Carfi et al., 1995), is adopted by various metallo-enzymes (Aravind, 1997). It consists of a four-layered β sandwich with two mixed β sheets flanked by α helices, with the metal-binding sites located at one edge of the β sandwich (Color Plate 13.V). Sequence analysis and secondary structure prediction for Artemis indicated conservation of motifs typical of the metallo-β-lactamase fold, participating in the metal-binding pocket and representing the catalytic site of the metallo-β-lactamases. Altogether, this analysis indicated that Artemis not only probably adopted the β-lactamase fold, but may also have conserved an associated catalytic activity (Aravind, 1997).

MUTATION ANALYSIS

Eight different alterations of Artemis were found in 11 families (Plate 13.VI); although some of the mutations were recurrent, it was not possible to draw any clear correlation with the geographical origins of the patients. One, the C279T modification, created a nonsense mutation. Other mutations, including amino acid substitutions, were subsequently described (Kobayashi et al., 2003; Li et al., 2002; Noordzij et al., 2003). The other nucleotide changes affected splice donor sequences leading to either frameshifts in three cases or to in-frame deletion of part of the protein in one case. The Artemis gene may represent a hot spot for deletion. Three mutations were deletions spanning several exons, leading to frameshifts and premature terminations in two cases and in-frame deletion of 216 amino acids in one case. In three patients a deletion comprised exons 1 to 4, resulting in a complete absence of Artemis mRNA. This null allele demonstrated that Artemis is not an essential protein for viability, in contrast to XRCC4 or DNA-ligase IV, for example (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998a).

Hypomorphic Artemis mutations were found in a patient affected by OS with clinical presentation as well as immunophenotype indistinguishable from those caused by hypomorphic mutations RAG mutations (Ege et al., 2005). Furthermore, hypomorphic mutations of Artemis that allow the emergence of a few B and T lymphocytes were accompanied by the development of EBV-associated B-cell lymphomas in a general context of genomic instability (Moshous et al., 2003). This is reminiscent of the pro-B cell lymphomas that emerge in NHEJ-deficient mice when crossed onto a cell-cycle checkpoint defect such as $p53^{-/-}$ (Ferguson et al., 2001).

Artemis knockout mice are viable and recapitulate the phenotype seen in human RS-SCID patients (Li et al., 2005; Rivera-Munoz et al., 2009; Rooney et al., 2002). In the model developed by Rooney et al., a significant numbers of bona-fide mature T lymphocytes, mostly $CD4^+$, are detected in the periphery of certain mice. This may be a consequence of the genetic background (Xiao et al., 2009). Artemis deficiency in mice results in chromosomal fragments, fusion, and detached centromeres in both embryonic stem cells and murine embryonic fibroblasts (Rooney et al., 2002, 2003). These findings strongly suggest that Artemis has an important role in genome stability and may be considered a genomic caretaker.

STRUCTURE AND FUNCTION

With no ortholog of human and murine Artemis in other species, we are left with the similarity of Artemis to various members of the metallo-β-lactamase family, including murine SNM1 and yeast PSO2. However, despite their SNM1 similarity regions, the three proteins differ in their associated domains. While murine and yeast SNM1/PSO2 mutants demonstrate a strong defect in the repair of DNA damage caused by DNA interstrand cross-linking agents (Dronkert et al., 2000; Henriques and Moustacchi, 1980), they do not display elevated sensitivity to ionizing radiation, indicating that these two proteins are probably not directly involved in the repair of DNA DSB. This is in sharp contrast to the phenotype of RS-SCID patients, whose primary molecular defect is indeed the absence of DNA DSB repair, illustrated by the lack of coding joint formation in the course of V(D)J recombination and the increased sensitivity of bone marrow and fibroblast cells to γ rays (Cavazzana-Calvo et al., 1993; Nicolas et al., 1998). Interestingly, Artemis, murine SNM1, and yeast PSO2 share a domain adopting a metallo-β-lactamase fold. Sequence analysis revealed the existence of a conserved region that accompanies the metallo-β-lactamase domain in members of the Artemis/SNM1/PSO2 subfamily, including various other sequences related to nucleic acid metabolism such as two subunits of the cleavage and polyadenylation specificity factor (CPSF). We named this domain βCASP, for metallo-β-lactamase-associated CPSF Artemis SNM1/PSO2 domain (Callebaut et al., 2002). It is tempting to speculate that this domain could contribute to substrate binding, in a way similar to the α-helical domain of glyoxalase, another member of the β-lactamase family (Cameron et al., 1999).

DNA DSBs can be repaired either by homologous recombination (HR) or by the NHEJ (reviewed in Haber, 2000). Whereas HR is the predominant repair pathway in yeast, NHEJ is mostly used in higher eukaryotes and represents the DNA repair pathway followed during V(D)J recombination. At least two protein complexes are thought to act in concert or sequentially at the site of the RAG1/2-derived DSBs. The Ku70–80 complex is probably recruited first at the site of the lesion, followed by the addition of the DNA-PKcs subunit. This initial complex is considered the primary DNA damage sensor that will activate the DNA repair machinery. The XRCC4/DNA-ligase IV complex represents the best candidate to actually repair the gap.

Careful analysis of the various phenotypes among the different V(D)J recombination deficient models, including RS-SCID, has provided some hypotheses regarding the possible role of Artemis during V(D)J recombination. Two major differences exist between the RS-SCID condition and that of XRCC4 and DNA-ligase IV knockout mice. First, a

complete null allele of Artemis does not lead to embryonic lethality in humans. This observation, therefore, does not support an implication of Artemis in this phase of NHEJ. Second, the rejoining of linearized DNA constructs introduced in RS-SCID fibroblasts is not altered (J. P. de Villartay, unpublished observations), whereas this assay, when defective, is highly diagnostic of abnormal NHEJ in yeast (Teo and Jackson, 1997; Wilson et al., 1997). Perhaps the most evident link between Artemis and NHEJ is found in regard to the Ku/DNA-PK complex. Indeed, human RS-SCID patients and *scid* mice, which harbor a mutation in DNA-PKcs, are the only conditions in which a V(D)J recombination-associated DNA repair defect uniquely affects coding joints, leaving signal joint formation unaltered. This is in striking contrast to all the other known V(D)J recombination/DNA repair deficiency settings. Hairpin-sealed coding ends represent the unprocessed V(D)J recombination intermediates that accumulate in murine *scid* lymphoid cells. Although recent data have indicated that RAG1 and RAG2 are capable of opening these hairpin structures in vitro, repair factors such as DNA-PK may be required for this process in vivo. Artemis, through its putative hydrolase activity, may participate in opening the hairpin at the coding ends in vivo. Artemis does indeed possess an intrinsic exonuclease activity in vitro that can be redirected to an endonuclease activity capable of resolving Rag1/2-generated hairpins in vitro when Artemis is complexed to and phosphorylated by DNA-PKcs (Ma et al., 2002). The catalytic core of Artemis is carried by the metallo-β-lactamase/βCASP domain (J. P. de Villartay, unpublished observations). The accumulation of hairpin-sealed coding ends in thymocytes from Artemis and scid/DNA-PKcs knockout mice strongly supports this function (Rooney et al., 2002).

Although it is clear that factors of the NHEJ are required to complete V(D)J recombination, the question of their role during another essential recombination phase of immunoglobulin genes, the class switch recombination (CSR), has been a matter of debate. Recently it was demonstrated that in the absence of a bona-fide NHEJ pathway (occurring in the lack of XRCC4 and DNA-ligase IV), CSR is only moderately affected (Soulas-Sprauel et al., 2007). Likewise, although Artemis was first thought dispensable for CSR (Rooney et al., 2005), the identification of unresolved CSR-generated DNA breaks in B cells from Artemis-deficient mice suggests a possible role for Artemis during CSR (Franco et al., 2008).

DNA-LIGASE IV DEFECTS

As previously described, DNA-ligase IV forms a complex with XRCC4, which is essential for NHEJ and V(D)J recombination (Critchlow et al., 1997; Grawunder et al., 1997). The inactivation of both alleles of DNA-ligase IV in a human pre-B cell line produced radiosensitivity and abrogated V(D)J recombination activity (Grawunder et al., 1998a). Riballo et al. (1999) identified a patient with a defect in NHEJ bearing a missense mutation in the gene encoding DNA-ligase IV. This patient did not show any immunodeficiency; however, he developed leukemia at age 14 and overresponded to radiotherapy (Riballo et al., 2001). More recently, the finding of *LIG4* mutations in four patients thought to have Nijmegen breakage syndrome (NBS) at clinical presentation led to the identification of a new syndrome, designated *LIG4 syndrome*. This disorder is characterized by developmental delay, chromosomal instability, and immunodeficiency (O'Driscoll et al., 2001).

The clinical features of these patients resemble those of NBS, but the cellular phenotype is distinct. Although cells from both syndromes show radiosensitivity, LIG4-deficient cell lines have normal checkpoint function and are defective in DSB repair. The analysis of chromosome breakage in peripheral blood lymphocytes obtained from LIG4 patients did not show any translocations or inversions involving chromosomes 7 and 14, which are typical of ataxia-telangiectasia and NBS.

Many of the features of patients with LIG4 syndrome resemble a particular form of dwarfism named *Seckel syndrome*, recently found to be due to AT and Rad3-related protein defects (O'Driscoll et al., 2003). Patients had microcephaly and one of them had a bird-like face. Pancytopenia is a common feature in these patients, but they do not develop a SCID phenotype, consistent with the hypothesis that the mutations impair but do not abolish the VDJ activity. Skin disease is common and includes plantar warts, psoriasis, and photosensitivity. None of these patients developed cancer, although two of four patients showed hypothyroidism and hypogonadism.

MUTATIONS

The first patient described by Riballo et al. (1999) who developed leukemia had a missense R278H mutation mapping to the LIG4 active site. The same mutation was also present in another patient whose clinical features were completely different, suggesting that epigenetic factors may influence the onset of the disease. Mutations Q280R, H282L, and M249E, also in the vicinity of the active site, resulted in impaired activity of the enzyme to a level of 5 to 10 percent. Two mutations in other patients were stop mutations (R580X, R814X) leading to protein truncation in the BRCT region, which is supposed to interact with XRCC4 (Grawunder et al., 1998b). It is noteworthy that LIG4-null mutations in mice result in embryonic lethality. This finding suggests that the mutations in these patients could be hypomorphic, thus explaining the mild phenotype of the immunodeficiency. However, the R580X stop mutation is not easily reconciled with this hypothesis. Deletion of 5 bp at nt position 1270–1274, causing a premature stop codon 20aa downstream K424, has been found in three patients exhibiting a SCID phenotype (Buck et al., 2006; Toita et al., 2007).

CERNUNNOS DEFECTS

Buck et al. (2006) reported patients with SCID, growth delay, and microcephaly who also had increased radiosensitivity,

defective V(D)J recombination, and impaired in vitro NHEJ activity reminiscent of LIG4 syndrome. However, neither LIG IV nor the other known NHEJ factors were found to be mutated (Revy et al., 2005). A new NHEJ factor named *Cernunnos* was identified through cDNA functional complementation of patients' fibroblasts. The same NHEJ factor, named XLF (for XRCC4-like factor), was independently identified through a yeast two hybrid screen using XRCC4 as bait (Ahnesorg et al., 2006). Deleterious mutations of the *Cernunnos* gene were found in all patients, and the ectopic expression of a wild-type *Cernunnos* complemented the DNA repair defect (Ahnesorg et al., 2006; Buck et al., 2006). *Cernunnos*-deficient murine ES cells had a phenotype similar to that of human deficient cells (increased radiosensitivity, genomic instability, defective DNA repair) but retained V(D)J recombination, although with decreased efficiency (Buck et al., 2006; Dai et al., 2003; Zha et al., 2007). In striking contrast with both XRCC4 and LIG4 knockout mice, *Cernunnos/XLF* deficiency does not result in embryonic lethality (Li et al., 2008, and J. P. de Villartay, unpublished observations). The immunological phenotype also appears less severe in these mice than in the human condition, with only a mild diminution of B and T lymphocytes in the periphery.

CERNUNNOS STRUCTURE

The human *Cernunno/XLFs* gene, composed of eight exons, is located on chromosome 2q35 and encodes a 2063 nucleotide transcript (Ahnesorg et al., 2006; Buck et al., 2006). The Cernunnos/XLF protein is 299 amino acids with an apparent weight of about 33 kDa. Although divergent, Cernunnos is the genuine ortholog of Nej1p/Lif2 (Callebaut et al., 2006), a NHEJ factor described in yeast (Frank-Vaillant and Marcand, 2001; Kegel et al., 2001; Valencia et al., 2001). XLF orthologs have been found in many eukaryotes (Callebaut et al., 2006; Cavero et al., 2007; Hentges et al., 2006). Nej1p in yeast interacts with the XRCC4 ortholog Lif1p, suggesting that Nej1p and Cernunnos have conserved an analogous function throughout evolution.

Cernunnos is ubiquitously expressed and localized predominantly in the nucleus. It shares structural features with *XRCC4*, revealing the existence of a new protein family (Ahnesorg et al., 2006, Callebaut et al., 2006; Hentges et al., 2006; Junop et al., 2000; Sibanda et al., 2001). The predicted conformation for Cernunnos (i.e., a globular head domain followed by a coil-coiled tail) has been confirmed (Andres et al., 2007; Li et al., 2008). Cernunnos, like XRCC4, can bind DNA in a sequence-independent manner (Hentges et al., 2006; Lu et al., 2007) and homodimerize or participate in the same complex together with LIG4 (Ahnesorg et al., 2006; Callebaut et al., 2006; Deshpande et al., 2007, Hentges et al., 2006). The exact nature of the complex(es) formed between XRCC4, DNA-ligase IV, and Cernunnos remains to be established. Despite their close relationship, Cernunnos and XRCC4 have distinct specific roles during NHEJ (Malivert et al., 2009).

CERNUNNOS FUNCTION

Like XRCC4 and several other factors that participate in the DNA damage response (DDR), Cernunnos and its yeast ortholog Nej1p are phosphorylated upon DNA damage (Ahnesorg et al., 2007; Wu et al., 2007). However, the recruitment of Cernunnos to the site of DNA breaks does not require this DNA-PK-dependent phosphorylation event (Wu et al., 2007). Although XRCC4 and Cernunnos share structural characteristics and are part of the same complex, overexpression of XRCC4 cannot functionally complement Cernunnos-deficient cells (Callebaut et al., 2006), suggesting that these two factors act in a cooperative manner. Moreover, defects of XRCC4 or Cernunnos have different impact on the LIG4, which is destabilized in the absence of XRCC4 (Grawunder et al., 1998; Teo et al., 2000) but not in Cernunnos-deficient cells (Ahnesorg et al., 2006; Callebaut et al., 2006). Although the XRCC4/LIG4 complex exerts DNA-end ligation in vitro (Grawunder et al., 1997), Cernunnos further potentiates this activity (Hentges et al., 2006; Lu et al., 2007). Cernunnos seems particularly important for the ligation of mismatched or noncohesive DNA ends, but not of compatible DNA ends, in vitro (Akopiant et al., 2009), suggesting that it may potentiate the ligation activity of the XRCC4/LIG4 complex on specific DNA end structures. Cernunnos is also involved in the response to replicative stress (Schwartz et al., 2009).

DNA-PKCS DEFICIENCY

Thus far, only one case of human DNA-PKcs deficiency has been reported. The patient presented with classical SCID with recurrent oral candidiasis and lower respiratory tract infections from the third month of life with progressive respiratory distress (van der Burg et al., 2009). She had minimal tonsillary tissue. She was successfully transplanted from a healthy HLA-identical male cousin without pretransplant conditioning.

MUTATION ANALYSIS

The above patient had two homozygous variations: a three-nucleotide deletion (c.6338_6340delGAG) resulting in the deletion of a Glycine (p.delG2113) and a missense mutation (c.9185T>G), resulting in replacement of one leucine with arginine (p.L3062R) (van der Burg et al., 2009). The missense mutation p.L3062R was the disease-causing mutation, as demonstrated with complementation assays (Fig. 13.2). The DNA-PKcs missense mutation did not result in an absence of protein and the mutated DNA-PKcs protein had retained autophosphorylation and kinase activity. The composition of the coding joints was suggestive of insufficient or inefficient Artemis activity, as Artemis activity depends on DNA-PKcs for phosphorylation. Similar to the *scid* mouse, the junctions showed an increased number of palindromic nucleotides, indicative of defective hairpin opening, as observed previously in Artemis-deficient patients (van der Burg et al.,

2007), although the number of P-nucleotides was lower in the DNA-PKcs-deficient patient. Accumulation of mutated DNA-PKcs protein to laser-induced DSB sites in living cells and subsequent recruitment of Artemis was normal. The DNA-PKcs mutation probably affects Artemis activation or Artemis positioning relative to the hairpin.

DNA-PKCS GENE STRUCTURE AND FUNCTION

DNA-PKcs is the catalytic subunit of the DNA-PK complex. It is a large protein of more than 4,000 amino acids and is a member of the PIKKs (phosphatidylinositol-3-kinase [PI3K]-like kinases) protein family (Abraham, 2004). Other members of this family that are involved in DNA damage response are ataxia-telangiectasia mutated (ATM) and AT-related (ATR) and RAD3 (see Chapters 47, 48). The N-terminal region consists of two HEAT (huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, TOR1) repeats (Brewerton et al., 2004) and a leucine-rich region (LRR), which has DNA-binding capacity (Fig. 13.2) (Gupta et al., 2005). The C-terminal region of DNA-PKcs is also conserved across the protein family. It is composed of a FAT (FRAP, ATM, and TRAP) domain, a PI3K kinase domain, and a C-terminal FATC domain. Ku80 has been shown to interact with the C-terminal region of DNA-PKcs. The C-terminus seems to mediate DNA-PKcs autophosphorylation, which in turn may influence DNA processing by Artemis (Weterings et al., 2009). Ku70/Ku80 can interact with DNA-PKcs only in the presence of DNA. The Ku70/Ku80 heterodimer binds specifically to double-stranded DNA ends in a ring shape and subsequently DNA-PKcs is recruited to form a DNA end synapsis, ensuring protection from exonuclease activities and juxtaposition of DNA ends (van Gent and van der Burg, 2007). The assembled DNA-PK complex then acquires the ability to phosphorylate a number of target proteins, including itself. This autophosphorylation is very important for the NHEJ reaction (Chan et al., 2002; Ding et al., 2003). DNA-PKcs has several autophosphorylation clusters, including the ABCDE cluster (T2609, S2612, T2620, S2624, T2638, and T2647) (Meek et al., 2004) and the PQR cluster (S2023, S2029, S2041, S2051, S2053, and S2056) (Cui et al., 2005; Meek et al., 2007). In addition, DNA-PKcs has an autophosphorylation site within the activation (T) loop of the kinase (T3950) (Douglas et al., 2007). The ABCDE cluster has a major role in remodeling the DNA-PK complex to make the DNA ends accessible for ligation (Block et al., 2004; Ding et al., 2003; Reddy et al., 2004), whereas phosphorylation in the PQR region appears to be involved in reducing access of nuclease activities to the DNA ends (Chen et al., 2005; Cui et al., 2005); phosphorylation of residue T3950 also influences the joining efficiency (Douglas et al., 2007). Although Ku70/80 binds DNA ends quite tightly, Ku70/80 as well as DNA-PKCS exchange from DSBs within a few minutes in living cells (Mari et al., 2006; Uematsu et al., 2007). The dynamics of NHEJ complexes is influenced dramatically by DNA-PK autophosphorylation: a DNA-PKCS mutant protein without kinase activity or with mutations in the autophosphorylation sites exchanged much more slowly than the wild-type protein (Uematsu et al., 2007).

DNA-PKCS ANIMAL MODELS

Spontaneous mutations in DNA-PKcs have been found in Arabian horses (McGuire et al., 1973), the classical *scid* mouse (Bosma et al., 1983), and Jack Russell terriers (Meek et al., 2001). All three species harbor mutations that result in absence of DNA-PKcs protein due to a C-terminal deletion (Fig. 13.2). In SCID horses, a 5bp deletion resulted in a frameshift and premature stop codon that prevented the translation of the 967 C-terminal codons (Shin et al., 1997; Wiler et al., 1995) (Fig. 13.2). C.B-17 BALB/c SCID mice have a nonsense mutation at codon 4045, predicting deletion of the 83 C-terminal amino acids (Blunt et al., 1996b). Jack Russell terriers also have a nonsense mutation that deletes the 517 C-terminal codons (Bell et al., 2002; Meek et al., 2001).

The phenotypes of horses, dogs, and the human SCID patient were similar. The phenotype in the mouse seems to be less severe than in the other species and is regarded as a "leaky" SCID. *Scid* mice are susceptible to bacterial, viral, fungal, and protozoal infections but can live beyond 1 year under germ-free conditions. This longer lifespan was explained by the presence of immunoglobulins and some T cells that apparently give some protection. DNA-PKcs is an evolutionarily conserved protein, which is highly homologous between these three species and human (≥80 percent homology at the protein level). However, the human L3062R mutation, which retained some kinase activity, differs substantially from the truncating mutations in SCID horses, mice, and dogs. In these three species,

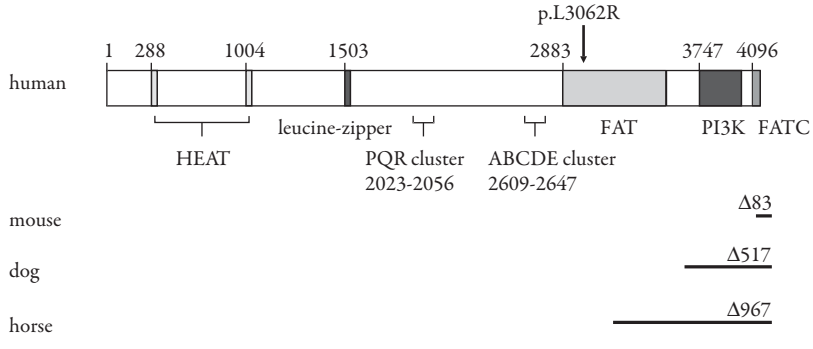

Figure 13.2 Schematic representation of the DNA-PKcs protein with the mutations as identified in human, mouse, dog, and horse. For details, see text.

the DNA-PKcs defects resulted in absence of protein and consequently in absence of kinase activity (Blunt et al., 1996a; Meek et al., 2001; Wiler et al., 1995). Low levels of mutated DNA-PKcs protein isolated from the *scid* mouse were shown to be catalytically inactive (Beamish et al., 2000).

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14.

IMMUNODEFICIENCY DUE TO DEFECTS OF PURINE METABOLISM

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INTRODUCTION

Genetic deficiency of the purine salvage enzyme adenosine deaminase (ADA) causes approximately 30–40 percent of autosomal recessive cases of severe combined immunodeficiency (SCID) and therefore approximately 15–20 percent of all cases of SCID (Bertrand et al., 1999; Buckley, 2000; Hirschhorn, 1979b). The identification of deficiency of adenosine deaminase as the basis for immunodeficiency (MIM 102700) was serendipitous and unexpected (Giblett et al., 1972). By contrast, immunodeficiency due to genetic deficiency of purine nucleoside phosphorylase (PNP, MIM 164050), the next enzyme in the purine salvage pathway, was then identified by specific screening for deficiency of enzymes in this pathway in immunodeficient patients (Giblett et al., 1975). PNP deficiency is significantly less common than ADA deficiency. Study of patients with these two disorders has shed light on the significance of the purine salvage pathway for lymphoid and nonlymphoid cells and led to the development of new antileukemic and immune-modulating agents (Dillman, 2004; Hershfield and Mitchell, 2001; Hirschhorn, 1993; Markert, 1994; Suetsugu et al., 1999).

Both ADA and PNP are ubiquitous, "housekeeping" enzymes whose deficiency essentially results in metabolic poisoning, with the most immediate effects manifested in lymphoid cells. As a result, both are diseases characterized by increasing attrition in immune function over time, as well as by blocks at specific steps of differentiation of lymphoid cells. Since the toxic metabolites that accumulate because of the enzyme defect primarily derive from dying cells, it is not surprising that clinical histories, particularly of patients with later onset, are consistent with the hypothesis that each infection results in additional attrition of immune cells and function. ADA and PNP deficiency share many clinical characteristics; however, several features distinguish the two diseases, reflecting varying expression and function of ADA and PNP enzymes (Table 14.1).

Since the previous edition of this book, several advances in both clinical and basic-science aspects of ADA and PNP deficiency have been made. Although the proportion of SCID cases due to ADA deficiency has not changed, only a few novel mutations were reported in recent years (Artac et al., 2009; Honig et al., 2007; Liu et al., 2009), suggesting either that this disease may be inherited from a restricted number of founders or that specific locations of the ADA gene are preferentially susceptible to mutagenesis. Following the initial report in 1996 of somatic reversion of an inherited ADA mutation to wild type (Hirschhorn et al., 1996), additional cases have been described (Liu et al., 2009), reflecting the recent increased awareness of somatic mosaicism due to in vivo reversion to normal of inherited mutations in several different immunological and nonimmunological disorders, including Wiskott-Aldrich syndrome (see Chapter 43) (Hirschhorn, 2003; Wada and Candotti, 2008). On the clinical side, studies have provided new insights into the molecular mechanisms causing T- and B-cell dysfunction and skeletal abnormalities in ADA-deficient patients. New phenotypic presentations, including non-infectious lung diseases with pulmonary alveolar proteinosis, Omenn syndrome, hematological findings of myeloid dysplasia, and susceptibility to dermatofibrosarcoma protuberans, have come into focus (Cassani et al., 2008; Roifman et al., 2008; Sauer et al., 2009; Sauer et al., 2012a; Sauer et al., 2012b, Booth et al., 2012, Grunebaum et al., 2012; Sokolic et al., 2011; Kesserwan et al., 2012). From the prospective of therapy and long-term outcome, it has been confirmed that hematopoietic cell transplantation (HCT) is often unable to improve or prevent the neurological abnormalities in long-term survivors (Honig et al., 2007) and that

Table 14.1 COMPARISON BETWEEN TYPICAL CASES OF ADA AND PNP DEFICIENCY

	ADA DEFICIENCY	PNP DEFICIENCY
Age of presentation	0.2–1 y	>1–2 y
Clinical manifestations at presentation	Infections Failure to thrive	Infections Neurological abnormalities Failure to thrive
Lymphopenia affecting	T and B cells (low NK cells)	T cells
Additional immune abnormalities	Autoimmunity Malignancy	Autoimmunity Malignancy
Organs affected	Bone marrow Liver Brain Skeleton Lung	Bone marrow Brain
Splenomegaly	Rare	Common
Reduced uric acid	No	Yes

HCT using mismatched donors is burdened by high mortality in this genetic form of SCID (Gaspar et al., 2009; Hassan et al., 2012). These findings have coincided with reports of efficacy of autologous hematopoietic cell gene therapy for ADA deficiency (Aiuti et al., 2009b; Gaspar et al, 2011, Sauer et al., 2012b, Candotti et al., 2012). Finally, the available *Ada* knockout mice have continued to prove as useful models for modeling and testing of cell and gene therapy approaches (Carbonaro et al., 2006, 2008, 2012), as well as contribute additional knowledge on nonimmunological consequences of ADA deficiency pointing to adenosine as an important profibrotic signal in the dermal skin and lungs (Chunn et al., 2005, 2006; Fernandez et al., 2008).

In PNP research, recognition of the importance of PNP substrates for T-cell development and function (Cohen et al., 1978) and determination of the human PNP protein structure (Ealick et al., 1990) have led to the development of several PNP inhibitors (Ravandi and Gandhi, 2006), some of which are being assessed for the treatment of T-cell lymphoma and autoimmunity (Gandhi et al., 2005; Morris and Omura, 2000). At the clinical level, several novel mutations in the *PNP* gene have been identified (Alangari et al., 2009; Al-Saud et al., 2009; Madkaikar et al., 2011; Walker et al., 2011; Somech et al., 2012) and early clinical observations that the nervous system is affected by PNP deficiency (Simmonds et al., 1987) have been complemented by demonstration of the important role of purine receptors in the brain (Liu and Salter, 2005). Finally, the availability of a mouse model of PNP deficiency that recapitulates most of the features observed in PNP-deficient humans (Arpaia et al., 2000) has provided a crucial tool for studying the pathogenesis of the immune and nonimmune abnormalities in PNP deficiency and facilitated the recent development of novel gene and protein replacement therapies (Liao et al., 2008; Toro and Grunebaum, 2006; Papinazath et al., 2011; Mansouri et al., 2012).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

ADA DEFICIENCY—CLINICAL SPECTRUM OF IMMUNODEFICIENCY

Early-Onset Disease

The early descriptions of clinical and pathological manifestations in ADA-deficient SCID patients were based upon retrospective studies of children diagnosed as having classical SCID with marked developmental delay and failure to thrive (Hirschhorn, 1979a; Meuwissen et al., 1975). Because of the original means of identifying patients to be tested for ADA deficiency, over 95 percent of the initial cases described were clinically and immunologically virtually indistinguishable from patients with other forms of classical SCID. These ADA-deficient patients had neonatal-onset disease with lymphopenia, absence of both cellular and humoral immune function, failure to thrive, and a rapidly fatal course due to infections with fungal, viral, and opportunistic agents (Hirschhorn, 1979a; Meuwissen et al., 1975). Many of these early-onset cases lacked B cells as well as T cells and would now be classified as having T cell-negative and B cell-negative (T^- B^-) SCID.

In some cases, ADA-deficient patients have presented with lymphadenopathy, hepatosplenomegaly, and erythroderma, thus mimicking the presentation of Omenn syndrome (Roifman et al., 2008). However, in addition to immunological defects, a distinguishing feature in approximately 50 percent of this group is a skeletal abnormality of the costochondral junctions, best visualized by X-ray on a lateral exposure of the chest as cupping and flaring, with a unique histology on biopsy (Cederbaum et al., 1976; Manson et al., 2013). A small percentage of cases, including the first two cases described, exhibit a somewhat delayed onset of disease, with diagnosis as late as the second year of life. Such patients may transiently retain the capability to produce autologous immunoglobulins, although both originally described patients produced essentially no specific antibodies (Table 14.2).

Delayed/Late-Onset Disease

It is now apparent that the clinical spectrum resulting from ADA deficiency is much broader than classical SCID. Testing for ADA deficiency in a wide range of individuals with abnormal immune function has broadened the spectrum considerably, not only as to age of onset but also as to immunological abnormality. Following the early identification of severely affected infants with ADA-deficient SCID and then the appreciation that onset could be somewhat delayed in the first two years of life and be associated with retention of immunoglobulin production, an increasing number of patients are now being found with late-onset ADA deficiency diagnosed between 3 to 15 years of age and even in adulthood (Table 14.2) (Antony et al., 2002; Artac et al., 2010; Geffner et al., 1986; Hershfield, 1998; Hirschhorn et al., 1993b, 1996; Levy et al., 1988; Morgan et al., 1987; Ozsahin et al., 1997; Shovlin et al., 1993). These later-onset patients present with significant

Table 14.2 CLINICAL PHENOTYPES IN ADA DEFICIENCY

1. Neonatal/infantile onset—Severe combined immunodeficiency	Clinically indistinguishable from other forms of SCID, except for bony abnormality in 50% of patients. May present as Omenn syndrome.
2. Delayed/late onset in childhood or adulthood—Combined immunodeficiency	Progressive attrition of immunity. May present with recurrent bacterial sinopulmonary infections, persistent viral warts or recurrent or severe varicella or herpes zoster; immune-mediated thrombocytopenic purpura (ITP). May have lymphopenia, elevated serum IgE, eosinophilia, autoantibodies.
3. ADA deficiency without immunodeficiency (partial)	Identification by population screening or analysis of relatives of affected individuals. No confirmed immunodeficiency to date.
4. ADA deficiency ameriolated by somatic revertant mutations or other function-restoring mutations resulting in lymphocyte mosaicism	Clinical improvement over time without therapy, lower-than-expected levels of toxic metabolites. and newly detected residual ADA enzyme activity

immunodeficiency but may have any of a variety of clinical histories, including recurrent sinopulmonary bacterial infections, episodes of pneumonia frequently due to *S. pneumoniae* with or without septicemia, and HPV disease. After infection or immunization these patients typically fail to produce antibody to some antigens, such as *S. pneumoniae* polysaccharides. They may lack IgG_2 antibodies but have markedly elevated IgE and/or eosinophilia. Immune dysregulation may be present, manifested by autoimmunity including autoimmune hypothyroidism, diabetes mellitus, hemolytic anemia, and idiopathic thrombocytopenia. All have had lymphopenia.

ADA Deficiency with Immunodeficiency Diagnosed in Adulthood

Similar to the ADA-deficient patients with late onset, the small number of adults who have been diagnosed with ADA deficiency have had variety of clinical presentations, including recurrent sinopulmonary bacterial infections, septicemia, and pulmonary infections. Persistent viral warts, recurrent herpes zoster attacks, and asthma have been noted, as well as autoimmune hypothyroidism, hemolytic anemia, and idiopathic thrombocytopenia. Initial immunological investigations have revealed absence of IgG_2, failure to produce antibody to pneumococcal antigens, elevated IgE, eosinophilia, and autoantibodies. Diagnosis of ADA-deficient immunodeficiency in adulthood can be complicated by effects of administration of immunosuppressive medications for autoimmune disorders. The autoimmune phenomena probably reflect abnormal regulation of immune responses, which has been related to loss of regulatory T cell function (Sauer et al., 2012a), as well as new defective B cell tolerance (Sauer et al., 2012b).

In the three initial published cases of ADA deficiency diagnosed in adulthood, lymphopenia was present prior to the diagnosis of immunodeficiency (Ozsahin et al., 1997; Shovlin et al., 1993). The first cases were two siblings with recalcitrant warts as a major manifestation, who in retrospect had the onset of immunological abnormalities during late adolescence (Antony et al., 2002; Shovlin et al., 1993). One of the sibs with severe lung disease died in her late 30s, despite enzyme replacement therapy (PEG-ADA, see "Treatment and Prognosis"). The surviving sib improved substantially with PEG-ADA but then developed a neutralizing antibody response to the enzyme sufficient to preclude continued therapy. At that time, treatment with ADA enzyme encapsulated into autologous erythrocytes, which represents the first therapeutic approach of this kind for ADA deficiency, was used (Bax et al., 2007). One additional patient was diagnosed at the age of 39 after a long history of multiple infections and leukopenia in childhood. In adult life she was well until septicemia followed a cesarean section. She also exhibited hepatic granulomas of unknown etiology and pulmonary tuberculosis as well as asthma and elevated IgE (Ozsahin et al., 1997).

It is likely that these adult patients with ADA defects constitute one subset of patients currently classified as having common variable immunodeficiency (CVID, see Chapter 23), although a screening of 44 unselected CVID patients failed to identify any individuals with ADA or PNP deficiency (Fleischman et al., 1998), suggesting that ADA-deficient subjects do not represent a large subset of CVID. Perhaps selection for such markers as early lymphopenia, hyper-IgE, eosinophilia, asthma, and persistent warts might be informative.

ADA Deficiency with Normal Clinical Phenotype ("partial" ADA deficiency)

An additional group of individuals with absence of ADA activity in erythrocytes has been identified by screening of normal populations or in healthy relatives of affected patients. Most of these individuals were identified as newborns through a screening program directed at early detection of ADA-deficient SCID patients. These patients were initially termed "partially deficient" because while they showed absent ADA activity in red cells, they retained 5 to 80 percent of normal ADA enzyme activity in nonerythroid cells (Borkowsky et al., 1980; Hirschhorn et al., 1979a, 1983, 1989, 1990, 1997; Hirschhorn and Ellenbogen, 1986). These children had dATP levels that were only marginally elevated and in amounts that are insignificant relative to values observed in immunodeficient patients. Although these children were healthy in early childhood, and one subject (homozygous for Ala215Thr mutations) was healthy as late as 18 years of age, their eventual outcomes are unknown. One of these children, lost to follow-up, carried a partial mutation

heterozygous with a "null" similar to adult-onset patients (Shovlin et al., 1993).

A number of healthy relatives in families with a proband with SCID were found to carry additional ADA mutations and to exhibit very low levels of ADA activity in blood cells (Ariga et al., 2001a; Ozsahin et al., 1997).

ADA DEFICIENCY—NONIMMUNOLOGICAL ABNORMALITIES

Several abnormalities have been described in only a few patients and therefore could reflect effects of infectious agents rather than primary defects due to ADA deficiency. These include renal and adrenal abnormalities, neurological abnormalities similar to those seen in PNP deficiency (see below), pyloric stenosis, and hepatic disease (Bollinger et al., 1996; Hershfield and Mitchell, 2001; Hirschhorn, 1979a; Ratech et al., 1985, 1989; Somech et al., 2009; and Hirschhorn, unpublished). In a series of 14 cases, morphological abnormalities of the myeloid lineages have been observed in the peripheral blood from 12 patients (>85 percent) and in the bone marrow of 5 out of 5 cases evaluated. These findings were associated with neutropenia in 7 patients (Sokolic et al., 2011) and suggest that ADA deficiency may have significant hematological consequences beyond affecting the generation of lymphoid lineages. Neutropenia and myeloid dysplasia have also been observed in a delayed-onset patient (Nomura et al., 2013). Abnormal platelet aggregation has also been described (Schwartz et al., 1978). Although it is difficult to prove that neurological abnormalities are not secondary to viral encephalitis, we have suggested that these abnormalities might reflect the interaction of high concentrations of adenosine with known adenosine A1 receptors in nervous tissue. This hypothesis is based upon our finding of amelioration of neurological manifestations concomitant with therapeutic measures that resulted in lowering of metabolites that otherwise accumulate in ADA deficiency (Hirschhorn et al., 1980a). Lymphoma, often associated with cells bearing Epstein-Barr virus (EBV) genomes, has occurred in several patients. In addition, a striking association between ADA deficiency and the occurrence of dermatofibrosarcoma protuberans has been recently demonstrated, with 8 of 12 patients evaluated in one series presenting with a previously unrecognized multicentric variant of this rare skin sarcoma (Kesserwan et al., 2012).

Several observations have demonstrated that ADA deficiency is frequently accompanied by cognitive and behavioral abnormalities, as well as neurosensorial deafness, that appear not to be shared by patients with other forms of SCID (Albuquerque and Gaspar, 2004; Rogers et al., 2001; Tanaka et al., 1996). Also at difference with other forms of SCID, ADA-deficient patients often present with pulmonary alveolar proteinosis (PAP) and severe respiratory symptoms and radiological findings in the absence of positive microbiological isolates (Booth et al., 2012; Grunebaum et al., 2012). These findings suggest a metabolic pathogenesis of the lung disease in ADA-defieincy that is reminiscent of the pulmonary abnormalities observed in *Ada* knockout mice (Blackburn et al., 2000) and deserves further investigation.

PNP DEFICIENCY—CLINICAL SPECTRUM OF IMMUNODEFICIENCY

Previously, it has been estimated that approximately 4 percent of patients presenting with symptoms of SCID have PNP deficiency (Markert, 1991). However, the variable clinical spectrum of PNP deficiency and the difficulties in diagnosing PNP-deficient patients prevent accurate determination of the true incidence and prevalence of this disease. To date, patients with PNP immunodeficiency from approximately 60 families have been reported worldwide (Al-Saud et al., 2009; Alangari et al., 2009; Andrews and Markert, 1992; Aust et al., 1992; Aytekin et al., 2010; Baguette et al., 2002; Banzhoff et al., 1997; Broome et al., 1996; Carpenter et al., 1996; Classen et al., 2001; Dalal et al., 2001; Dror et al., 2004; Madkaikar et al, 2011; Markert, 1991; Markert et al., 1997; Moallem et al., 2002; Pannicke et al., 1996; Sasaki et al., 1998; Somech et al., 2012; Tam and Leshner, 1995; Tsuda et al., 2002; Yamamoto et al., 1999). Additional patients have been identified but not reported (Roifman, unpublished).

The most characteristic immune abnormality observed in PNP-deficient patients is a profound T-cell defect resulting in SCID. Patients typically present in the first or second year of life with recurrent upper and lower respiratory tract infections due to bacterial, viral, or opportunistic pathogens. PNP-deficient patients also often fail to thrive. Debilitating meningitis infections were reported among some patients (Chantin et al., 1996). Urinary tract infections are frequent among PNP-deficient patients (Sasaki et al., 1998; Watson et al., 1981) but uncommon in other forms of SCID. Additional patients have been described who did not present with infections until later in childhood (Fox et al., 1977). Physical examinations often reveal small lymph nodes and tonsils (Rich et al., 1979), while diagnostic imaging studies, such as chest X-rays and computed tomography or dedicated ultrasound exams of the neck, show a small or absent thymus (Staal et al., 1980b).

The profound T-cell abnormality also predisposes PNP-deficient patients to contract disseminated and persistent varicella zoster infections that are particularly difficult to treat (Baguette et al., 2002; Banzhoff et al., 1997; Classen et al., 2001; Dror et al., 2004; Hallett et al., 1994; Sakiyama et al., 1989; Simmonds et al., 1987). One patient died at 9 years of age from demyelinating progressive multifocal leukoencephalopathy caused by JC virus (Parvaneh et al., 2007). Although the hallmark of PNP immunodeficiency is a profound susceptibility to pathogens controlled by T cells, only a single case of fatal pneumonia due to *Pneumocystis carinii* and Legionella infection has been observed (McGinniss et al., 1985), and only one patient has been reported to die from measles infection (Baguette et al., 2002). Moreover, many PNP-deficient patients tolerated bacillus Calmette-Guérin (BCG) immunizations, as well as live attenuated polio and MMR vaccinations (Markert, 1991), although measles virus was detected in the lungs, liver, and spleen of one patient after MMR immunization (Grunebaum and Roifman, 2002). Thus, while PNP deficiency causes a severe T-cell immunodeficiency, patients seem to be less susceptible, at least early in life, to opportunistic infections than in other forms of SCID.

Reduced B-cell numbers and abnormal humoral functions have been reported in a few PNP-deficient patients (Markert et al., 1987) and lack of B cells containing signal joint circles and abnormal BCR repertoires have also been reported, pointing to direct negative effects of PNP deficiency on B lymphocytes (Somech et al., 2012). In addition, defective production of antibodies has been documented in many PNP-deficient patients (Giblett et al., 1975; Hershfield and Mitchell, 2001; Markert, 1991; Somech et al., 2012). One patient developed chronic meningoencephalitis due to echovirus infection (Markert, 1991), a complication more often seen among patients with X-linked agammaglobulinemia.

Autoimmunity is common among PNP-deficient patients. In a large review, 13 of 34 PNP-deficient patients had one or more autoimmune disorders (Markert, 1991). Autoimmune manifestations included noninfectious arthritis, pericarditis, autoimmune thyroiditis, lupus-like symptoms, central nervous vasculitis, and massive splenomegaly (Carpenter et al., 1996; Parvaneh et al., 2007; Rich et al., 1979; Tam and Leshner, 1995). Severe immune-mediated hematopoietic cytopenias can be extremely difficult to treat in PNP-deficient patients (Carapella De Luca et al., 1986; Delicou et al., 2007; Markert et al., 1987; Rich et al., 1979). It is also important to distinguish between cytopenias caused by autoimmunity and those caused by primary bone marrow abnormalities (see below).

Abnormal immune surveillance in PNP deficiency likely contributes to the increased incidence of uncontrolled cell proliferations observed in these patients. One patient had monoclonal gammopathy (Rich et al., 1979), another died of hemophagocytic lymphohistiocytosis (Grunebaum and Roifman, 2002), and several have developed lymphoma (Banzhoff et al., 1997; Blatt, 1990; Soutar and Day, 1991). One PNP-deficient patient had a malignant pharyngeal tumor.

PNP DEFICIENCY—NONIMMUNOLOGICAL ABNORMALITIES

PNP is a ubiquitous enzyme required for purine metabolism in all cells; thus, it is not surprising that many PNP-deficient patients also suffer from nonimmunological abnormalities. Neurological dysfunction, which precedes and cannot be explained by the immune abnormalities, is found in more than half of the patients (Markert, 1991). The majority of neurological manifestations are related to motor development and function, including nonprogressive cerebral palsy, spastic paresis, hypotonia or hypertonia, difficulties maintaining posture, tremor, and ataxia (Al-Saud et al., 2009; Alangari et al., 2009; Aytekin et al., 2008; Baguette et al., 2002; Broome et al., 1996; Carpenter et al., 1996; Dror et al., 2004; Gelfand et al., 1978; Madkaikar et al., 2011; Rich et al., 1979; Simmonds et al., 1987; Staal et al., 1980b; Stoop et al., 1977; Watson et al., 1981). Some patients also suffer from varying degrees of hyperactivity, behavioral problems, deafness, blindness, and mental retardation (Sakiyama et al., 1989). Systematic evaluations of the neurological abnormalities have not been reported, and the few reports of cranial magnetic resonance imaging (MRI) performed in PNP-deficient patients provide conflicting data. A 26-month-old girl with retarded cognitive and motor development was found to have mild cerebral atrophy and bilateral hypomyelinization in the globus pallidus and periaqueductal area (Ozkinay et al., 2007). An MRI performed in a 3-year-old child who suffered from spastic paraplegia from the first year of life showed abnormal hypersignals in the white matter located behind the occipital horns and in the centrum semiovale (Tabarki et al., 2003). Interestingly, her sister, who suffered from similar neurological abnormalities, had normal MRI studies at 8 and 20 months of age. Abnormal motor nerve conduction velocity was also reported in one patient (Tabarki et al., 2003). Importantly, in contrast to the neurological defects that occur in other inherited immune deficiency diseases, the neurological abnormalities in PNP deficiency often precede infections or autoimmunity. Thus, association of severe T-cell immune deficiency with noninfectious nervous system abnormalities should suggest the diagnosis of PNP deficiency.

A few PNP-deficient patients develop bone marrow abnormalities, possibly related to an increased sensitivity of the bone marrow cells to oxidative damage (Carpenter et al., 1996; Delicou et al., 2007; Dror et al., 2004; Soutar and Day, 1991; Staal et al., 1980b; Stoop et al., 1977; Watson et al., 1981). Decreased proliferation of skin fibroblasts from PNP-deficient patients has also been reported, although the clinical significance of this finding is still not clear (Burke et al., 1977).

PATHOLOGY

ADA Deficiency. Examination of tissues at autopsy has been reported only in ADA-deficient patients with early-onset "classical" SCID. Abnormalities in the spleen, lymph nodes, gut, and thymus primarily reflect an absence of cells of the lymphoid system (Hirschhorn, 1979b; Ratech et al., 1985, 1989). Thymic pathology, examined by biopsy as well as autopsy, demonstrates an absent or small dysplastic organ with sparse, if any, lymphocytes; this is seen in most SCID cases and is not specific for ADA deficiency. Although florid pathology in this group of patients is predominantly limited to the immune system, some nonlymphoid organs also show unusual features (Hirschhorn et al., 1982; Mills et al., 1982; Simmonds et al., 1978). Thus, approximately 50 percent of early-onset ADA-deficient SCID patients exhibit a radiologically detectable bony lesion accompanied by a histological appearance unique to ADA deficiency (Cederbaum et al., 1976; Manson et al., 2012). While the lesion may be pathognomonic histologically, we have shown that the radiological abnormality is not specific for ADA-deficient SCID and can be seen in other disorders (Hirschhorn et al., 1979b). Nonetheless, the correct diagnosis of ADA-deficient SCID has not infrequently been suggested by the characteristic appearance of flared costochondral junctions on routine chest X-ray, best appreciated on a lateral view, as well as by the physical finding of a "rachitic rosary." Abnormalities of renal function have been noted in some patients, and we have described an unusual mesangial sclerosis in autopsy material from six of eight patients (Ratech et al., 1985). Renal abnormalities have also been described

in the murine model for ADA deficiency, suggesting that his abnormality is due to the primary metabolic defect itself. Additionally, we have described in these same patients an unusual form of adrenal cortical fibrosis. However, in view of the overwhelming and multiple infections in these children, the significance of the renal and adrenal lesions remains to be evaluated by comparison with autopsy material from SCID patients who were not deficient in ADA. In a patient with neonatal onset of disease, hepatic pathology was reported, with early giant-cell transformation, enlarged foamy hepatocytes, and portal and lobular eosinophilic infiltrates (Bollinger et al., 1996). In another early-onset case, elevated liver transaminases were noted at 6 weeks of life and the patient progressed to hepato-renal failure in the next 4 weeks with extensive microbiology investigations failing to identify infectious causes of hepatitis (Kühl et al., 2011). Although liver abnormalities were not appreciated in autopsies of the above eight patients with similar disease onset, the exclusion of known pathogens in these reported cases makes it possible that liver hepatitis may be a primary result of ADA deficiency.

Both histological and clinical evidence for graft-versus-host disease have been commonly reported at the time of diagnosis because of maternal T-cell engraftment or the administration of unirradiated blood products containing HLA-incompatible lymphocytes that attack the patient's tissues. Graft-versus-host reactions are fortunately now a rare complication of transfusions, but they can still be observed after transplantation therapy.

PNP Deficiency. Pathological reports are available from few PNP-deficient patients. An autopsy of a 23-month-old girl who suffered from familial disequilibrium-diplegia and T-lymphocyte deficiency showed atrophic lymph nodes with scant lymphocytes in the paracortical zone (Graham-Pole et al., 1975). Germinal centers could not be detected, although plasma cells were present in normal numbers. In addition, lymphocyte depletion was noted in the periarteriolar zones of her spleen. The status of the thymus was not recorded. The cerebellum was of normal size, but histological examination of the cerebellum was not performed. Appendectomy and hemicolectomy of her 7-year-old PNP-deficient brother also showed a paucity of submucosal lymphoid tissue and a marked reduction of lymphocytes, lack of germinal centers, but preserved plasma cells.

An atrophic thymus was found at autopsy of a 2.5-year-old PNP-deficient boy who died of malignant histiocytic lymphoma after treatment with prednisone and vincristine. Histological assessment showed dysplastic changes, lymphocyte depletion, epithelial immaturity, an absence of well-formed Hassall's corpuscles and lack of corticomedullary differentiation (Watson et al., 1981). An autopsy of another PNP-deficient patient who died of disseminated viral infection, presumably varicella, after 9 months of bovine thymosin F5 infusions and an additional 9 months of uridine treatment, showed similar findings (Ammann, 1978). Microscopic study of the thymus, which was difficult to locate, revealed fatty infiltration, vague corticomedullary distinction, a reduced number of lymphocytes, increased numbers of spindle cells, and numerous poorly formed Hassall's corpuscles of variable size. The tonsils and lymph nodes were small, with poor organization and no well-formed germinal centers. Plasma cells were abundant. Many macrophages showed erythrophagocytosis. The appendix contained only a few round cells in the lamina propria. The spleen showed a marked decrease in white pulp mass, as well as congestion and focal necrosis. The autopsy of another 2-year-old PNP-deficient patient who developed neurological deficits at 3 months of age and suffered from profound tonus abnormalities did not reveal any structural brain lesion; however, histological examinations were not reported (Simmonds et al., 1987).

LABORATORY FINDINGS

IMMUNOLOGICAL FINDINGS

ADA Deficiency. Lymphopenia and attrition of immune function over time are the two findings common to all presentations of immunodeficiency due to ADA deficiency (Table 14.3). Lymphopenia, as well as elevation of toxic metabolites, such as deoxy ATP (dATP) in red blood cells, are already present prenatally and at birth, as has been demonstrated in affected pregnancies of families with prior children with early-onset ADA deficiency (Hirschhorn et al., 1980b; Linch et al., 1984). In early-onset patients, there is essentially complete absence of lymphocytes and of both cellular and humoral immune function. Isoagglutinins are generally absent; immunoglobulins, particularly IgA and IgM, are low to absent, although absence of IgG, and occasionally other isotypes, is not easily evaluated because of maternally derived IgG and variable amounts of immunoglobulins transiently produced by infants. Antibody responses to T-dependent antigens are severely depressed. In ADA-deficient patients with delayed presentation, B cells and antibodies may be found, but eventually all functional antibodies are lost, and a mono-oligoclonal distribution of immunoglobulin may be detected. Abnormal laboratory results seen in autoimmune states are another feature of later-onset patients.

PNP Deficiency. PNP deficiency has a profound effect on T-cell maturation and function. Most patients are lymphopenic, with markedly depressed T-lymphocyte numbers. T-cell receptor excision circles (TRECs), which represent new thymic emigrants, are also reduced (Somech et al., 2012 and Roifman, unpublished). Kappa-deleting recombination excision circles (KRECs), that are present solely in newly derived bone marrow B-cells have been also found to be decreased (Somech et al., 2012).

The function of lymphocytes from PNP-deficient patients, assessed by the ability to proliferate in response to mitogens, antigens, or foreign cells in vitro, is markedly reduced (Gelfand et al., 1978; Somech et al., 2012). Similarly, in vivo determinations of T-cell function, such as reactivity to Candida or PPD skin tests, are depressed. Importantly, T-lymphocyte numbers and function may be normal early in life and deteriorate as toxic purine metabolites accumulate and infections become more frequent (Gelfand et al., 1978; Markert, 1991). In addition, fluctuations in T-cell immunity have been reported (Markert et al., 1987). Therefore, repeated immune evaluations should

Table 14.3 COMMON LABORATORY ABNORMALITIES IN ADA-DEFICIENT INDIVIDUALS WITH IMMUNODEFICIENCY

TEST	CLINICAL PRESENTATION PHENOTYPES		
	INFANTILE ONSET	LATE (CHILDHOOD) ONSET	ADULT ONSET
ADA Enzyme Activity			
In erythrocytes	<1%	<1%	<1%
In lymphocytes	0.5%	0.5–3%	3-?%
SAH hydrolase, erythrocytes	10%	10%	?*
Metabolites[a]			
Erythrocyte dATP[b]	327–2,248	174–247	100–150
Lymphocyte dATP[c]	1,150	?	?
Urinary dAdenosine[d]	442–1,500	54–270	?
Urinary Adenosine[d]	29.4 ± 5.7		
Plasma dAdenosine (μM)	0.6–2		
Plasma Adenosine (μM)	1–6		
Immunological Tests			
Lymphopenia	++++	++	++
CD3 lymphocytes	Absent to trace	Markedly diminished	Markedly diminished
CD4/CD8 ratio	Too few cells to test	Often reversed	Reversed
Eosinophilia	Rare	Frequent	Frequent
Proliferation to PHA in vitro	Absent to markedly reduced	Absent to markedly reduced	Absent to markedly reduced
Proliferation to allogenic cells in vitro	Reduced	?	?
Proliferation to antigen in vitro	Absent to trace	Trace	Trace
Immunoglobulins in serum	Maternal IgG	Low to absent (absent IgG2)	Normal total levels; (low IgG2)
Serum IgE elevation	Not reported	Frequent	Frequent
Specific antibody responses in vivo	Absent	Absent to very low	?
Infections	Predominantly viral, fungal, and opportunistic; also bacterial sepsis	Increasing proportion of bacterial sinopulmonary	Bacterial sinopulmonary; herpes zoster, herpes simplex; candida
Other Clinical Abnormalities	50% costochondral junction abnormality	Autoimmunity; possibly increased asthma incidence	Autoimmunity; possibly increased asthma incidence

*Data not available.

[a]Metabolite concentrations based on R. Hirschhorn (published and unpublished data; see refs. In Hirschhorn et al., 1997, and Morgan et al., 1987)

[b]nmol/mL packed erythrocytes

[c]pmol/10^6 cells (Hirschhorn, 1992 et al.)

[d]nmol/g creatinine, range or average ± SD; normal adenosine: 4.12 ± 1.0; normal deoxyadenosine ≤0.1

be performed when PNP deficiency is suspected. Humoral immunity, assessed by immunoglobulin levels and the formation of specific antibodies to proteins and polysaccharide antigens, is normal in many, but not all patients (Ammann, 1978; Gelfand et al., 1978; Rich et al., 1979; Stoop et al., 1977). In some PNP-deficient patients, B-cell numbers and isohemagglutinins are reduced and the production of specific antibodies is poor (Somech et al., 2012). Two patients immunized with the T-cell-dependent bacteriophage ΦX174 responded with markedly depressed antibody titers, poor amplification, and a complete inability to switch from IgM to IgG. Abnormal antibody responses may represent defective interaction between T and B cells, or indicate a direct toxic effect of purine metabolites on B lymphocytes. Elevated IgE concentrations have also been reported (Ammann, 1978). One patient was found to have significantly increased serum concentrations of IL-18, possibly secondary to abnormal regulation of macrophages and NK cells (Yamamoto et al., 1999). Neutropenia, secondary to either autoimmune cytopenia or abnormal bone marrow function, is also common among PNP-deficient patients (Delicou et al., 2007; Dror et al., 2004; Soutar and Day, 1991).

BIOCHEMICAL FINDINGS

Protein Expression and Enzymatic Activity

ADA enzyme activity in cells from patients with classic ADA-deficient SCID is essentially undetectable (Table 14.3).

The reported activity must take into account the minor activity of a nonrelevant isozyme that exhibits ADA activity but is encoded by another genetic locus not affected in ADA-deficient SCID (Hirschhorn and Ratech, 1980; see "Biochemistry of ADA" below). Late-onset patients may retain 2 to 5 percent of normal activity, with the highest residual activity reported in subjects with adult-onset disease (Shovlin et al., 1993). In most patients examined for protein with anti-ADA-specific antibodies, ADA protein has been undetectable, suggesting that most ADA mutations result in unstable proteins (Arredondo-Vega et al., 2001; Otsu et al., 2002; Wiginton and Hutton, 1982). However, it is possible that the antibodies used did not recognize some mutant proteins.

Our own unpublished experiments using quantitative immunoprecipitation with a polyclonal antibody and a series of mutants did detect protein in quantities proportional to the relative enzyme activity. One patient originally reported to have normal protein for the amount of enzyme activity seen was demonstrated to be a somatic mosaic, with some cells expressing normal ADA (Hirschhorn et al., 1994b; see "Molecular Biology of ADA" below).

As mentioned, partially ADA-deficient individuals lack ADA activity in erythrocytes but express 5 to 80 percent normal activity in nonerythroid cells, such as cultured EBV-transformed B cells. These individuals were ascertained as a result of population screening undertaken in both normal adults and newborns (Hirschhorn, 1979a; Hirschhorn et al., 1990; 1997). They have been designated "partial ADA deficients" and to date have not presented with immunodeficiency. However, one of the same mutations that permits approximately 5 percent of normal activity has recently been identified in patients with adult-onset immunodeficiency, suggesting that some of the mutations present in partially ADA deficients may not be benign when hetero-allelic with a mutation that totally abolishes activity (see "Genotype–Phenotype Correlations" below).

The biochemical hallmark of PNP deficiency is markedly reduced or absent PNP enzymatic activity in cells. PNP enzyme activity is commonly measured in erythrocyte lysates by the conversion of radiolabeled inosine to hypoxanthine. The two metabolites are separated by thin-layer chromatography and quantified by a scintillation counter. Results are expressed in relation to time and the number of cells analyzed, the amount of protein in the sample, or the patient's hemoglobin. Thus, the PNP activity in erythrocyte lysates of normal individuals had been reported as 1,336 ± 441 nmol/hour/mg protein (Myers et al., 2004), 2,499 to 3,735 nmol/minute/10^6 cells (Dalal et al., 2001), or 2,700 to 3,300 nmol/hour/mg hemoglobin (Parvaneh et al., 2008). PNP activity can also be measured by a nonradiochemical HPLC-linked method, which has the advantage of allowing screening of PNP activity in dried blood spots (Jacomelli et al., 2002). PNP activity can also be quantified by spectrophotometery using coupled conversion of inosine to uric acid in the presence of xanthine oxidase (Chu et al., 1989; Hershfield and Mitchell, 2001; Osborne, 1980). By this assay, the mean normal erythrocyte PNP activity has been determined to be 18.6 U/mL of packed erythrocytes. PNP itself can be detected by Western blot analysis, as the protein typically migrates to the 32 kDa weight in denaturing conditions (Sasaki et al., 1998; Toro and Grunebaum, 2006); however, antibodies to human PNP are available only through a few research labs. Moreover, even if the protein is present, it may not function appropriately. Similarly, while indirect immune fluorescence is a rapid method for visualizing PNP in single cells (Pruslin and Reem, 1980), it may falsely detect nonfunctional enzyme.

Measurement of PNP activity using erythrocytes is affected by recent blood transfusions. As an alternative, PNP activity can be tested in isolated mononuclear cells or peripheral T cells, although the low number of lymphocytes and small blood volume obtainable from young patients often limits the number of cells that can be obtained.

PNP activity varies in different human cells and tissue extracts (Carson et al., 1977). Highest PNP levels are found in peripheral blood granulocytes and lymphocytes (121 and 115 nmol/minute/mg, respectively) followed by cells isolated from the kidneys, small intestine (100 and 64 nmol/minute/mg, respectively), and spleen (54 nmol/minute/mg). PNP activity in the liver, lung, and heart is practically identical and ranges between 32 and 38 nmol/minute/mg, while activity in the brain is the lowest, at 10 nmol/minute/mg. The activity in the thymus is 23 nmol/minute/mg, with higher activity in medullary thymocytes than cortical thymocytes. Another study demonstrated that PNP activity in skin fibroblasts was only 12 ± 2 nmol/minute/mg protein (Burgio et al., 1993). It should be noted that different laboratories have found different PNP activities in similar cells, emphasizing the importance of establishing a range in each lab and choosing appropriate controls.

Metabolic Consequences of ADA Deficiency

ADA is an enzyme of the purine salvage pathway that catalyzes the deamination of adenosine and 2-deoxyadenosine, as well as several naturally occurring methylated adenosine compounds (Hirschhorn and Ratech, 1980; Ratech et al., 1981, 1982). The deamination of adenosine and deoxyadenosine gives rise, respectively, to inosine and to deoxyinosine (Fig. 14.1). In humans these compounds can be further converted into hypoxanthine and then either enter a nonreversible pathway to uric acid or, by the initial action of hypoxanthine phosphoribosyl transferase (HPRT), be salvaged back from hypoxanthine into other purines. The absence of ADA would be expected to result in diminution of the products of the reaction, inosine and 2-deoxyinosine. However, the presence of alternative "bypass" pathways apparently results in normal concentrations of these two products in patients with ADA deficiency and, in contrast to PNP deficiency, also normal concentrations of uric acid, the final product of the pathway. Conversely, the absence of the ADA would be expected to result in accumulation of the substrates adenosine and 2-deoxyadenosine. Not only are these substrates found in increased amounts in plasma and excreted in urine, but they also spill over into additional pathways that are normally only minimally utilized (Cohen et al., 1978; Coleman et al., 1978; Fairbanks et al., 1994; Hirschhorn et al., 1981; 1982;

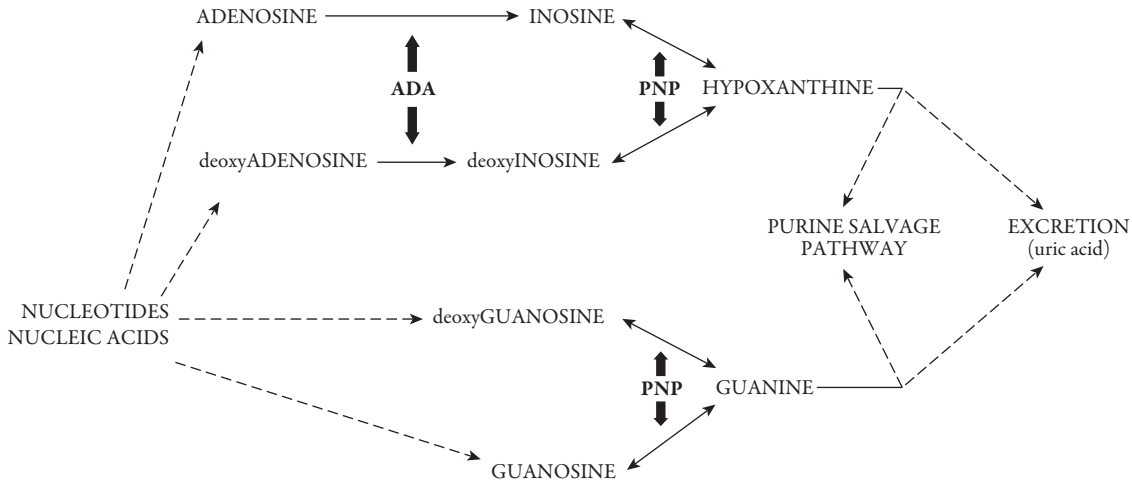

Figure 14.1 Schematic representation of ADA and PNP metabolic pathways.

Kuttesch et al., 1978; Mills et al., 1982; Morgan et al., 1987; Simmonds et al., 1978, 1982).

Deoxyadenosine is a component of DNA and primarily derives from the breakdown of DNA (Figs. 14.1 and 14.2). Therefore, 2-deoxyadenosine would be expected to be in highest concentrations at sites of frequent cell death such as the thymus, where lymphocytes undergo apoptotic death during the course of differentiation and repertoire selection. Adenosine is a component of adenine nucleotides, including ATP and RNA, and can be made both from the normal intracellular breakdown of ATP and from degradation of RNA following cell death. Both adenosine and deoxyadenosine are normally present in plasma, with normal concentrations of approximately 1 μM for adenosine and 0.5 μM for deoxyadenosine.

Patients with ADA-deficient SCID have elevated concentrations of adenosine and 2-deoxyadenosine in plasma (1 to 6 μM for adenosine, 1 μM for deoxyadenosine). The actual concentrations in plasma are difficult to determine by ordinary procedures, as both compounds are almost instantaneously taken up by erythrocytes during drawing of a blood sample and trapped intracellularly by phosphorylation. The most striking alteration in ADA deficiency is the accumulation of massive amounts of dATP in erythrocytes and lymphocytes (Hirschhorn et al., 1992). This results from uptake of the increased deoxyadenosine present in surrounding body fluids, with subsequent intracellular phosphorylation and trapping. In cases with the most marked accumulation of dATP, ATP concentrations fall. The accumulation of dATP in a particular cell type or tissue depends on the rate of phosphorylation versus the rate of dephosphorylation. There has been only one published study of dATP in nonlymphoid tissues (in a patient dying of lymphoma and previously treated with partial exchange transfusions). This study of the heart, brain, liver, and spleen reported only modest elevations in dATP, recalculated as 2 to 20 versus the 500 to 2,000 found in red blood cells (Coleman et al., 1985). Surprisingly, among several

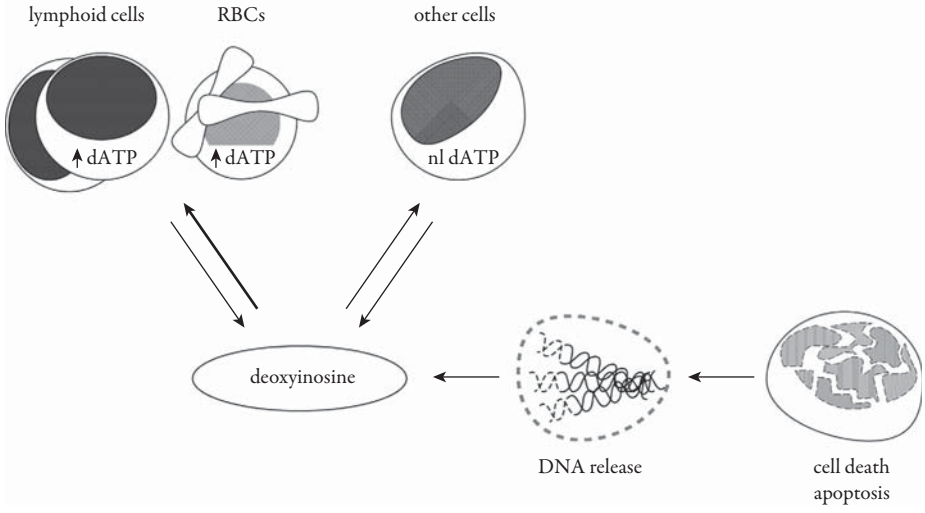

Figure 14.2 ADA deficiency: cellular and metabolic interactions.

tissues examined, massive accumulation of phosphorylated deoxyadenosine was found only in the kidney (R. Hirschhorn, unpublished studies).

Patients with ADA deficiency additionally show a massive increase in excretion of deoxyadenosine in urine, where it is normally undetectable. By comparison, excretion of adenosine is only minimally elevated over that found in normals. Several naturally occurring methylated adenosine compounds that are substrates for ADA are also excreted in urine in increased amounts in ADA-deficient individuals (Hirschhorn et al., 1982).

The increased concentrations of deoxyadenosine also inactivate the enzyme S-adenosylhomocysteine hydrolase (SAH hydrolase), resulting in a secondary deficiency of that enzyme activity in erythrocytes (Hershfield et al., 1979; Hershfield and Mitchell, 2001). Normalization of SAH hydrolase activity appears to be the most sensitive indicator of normalization of metabolite concentrations (see "Treatment and Prognosis" section below). Although several other alterations in metabolites have been proposed, based upon in vitro investigations of proposed pathophysiological mechanisms, none of these additional alterations has been demonstrated in vivo.

In general, adenosine metabolite concentrations correlate with severity of disease. In partially ADA-deficient individuals who are healthy, metabolite concentrations are on average greater than normal but still markedly lower than those found in immunodeficient patients. Red blood cell dATP in partially deficient subjects ranges from a normal of 5 to 40 nmol/ml, compared to 25-fold increased in adult-onset patients and over 1,000 fold-increased concentrations in infantile-onset patients (Table 14.3) (Hirschhorn et al., 1982, 1997). Deoxyadenosine excretion parallels concentrations of dATP, while adenosine concentration in urine is a poor discriminator between different types of individuals (Hirschhorn, unpublished).

Different therapeutic maneuvers lower the concentrations of metabolites to differing degrees. Following bone marrow transplantation, metabolites in red cells, plasma, and urine are dramatically decreased. However, sensitive measurements indicate that metabolites are still elevated, and in particular that plasma adenosine remains very high. Whether or not this is influenced by the bone marrow donor being a carrier or homozygous normal for the disorder is unknown. Metabolite concentrations following polyethylene glycol (PEG)-ADA therapy (see "Treatment and Prognosis") are lower with respect to dATP in red cells than seen following bone marrow transplantation or vigorous partial exchange transfusion (Hirschhorn et al., 1980a, 1981). It is not surprising that adenosine concentrations are less diminished, since the affinity of ADA is greater for deoxyadenosine than for adenosine.

Metabolic Consequences of PNP Deficiency

PNP (EC 2.4.2.1) catalyzes the reversible phosphorolysis of four purine nucleosides, guanosine, deoxyguanosine, inosine, and deoxyinosine, to yield guanine or hypoxanthine and ribose-1-phosphate or 2′-deoxy ribose-1-phosphate (Fig. 14.1). The purine bases, hypoxanthine and guanine, may then be recycled by the action of HPRT to inosine monophosphate (IMP) and guanosine monophosphate (GMP), which can undergo further phosphorylation to GTP. Alternatively, purine bases may be excreted as uric acid by the action of xanthine oxidase. The reverse reaction is not likely to occur in vivo because of the low levels of deoxyribose-1-phosphate and the rapid metabolism of hypoxanthine and guanine by HPRT.

PNP deficiency results in an accumulation of its substrates, guanosine, deoxyguanosine, inosine, and deoxyinosine, in all cells and body compartments, including cerebrospinal fluid (Hershfield and Mitchell, 2001). These metabolites can be accurately assessed by HPLC of cells, serum, urine, and even dried blood spots (Chantin et al., 1996). Deoxyguanosine may undergo phosphorylation by deoxyguanosine kinase (dGK) to dGMP and subsequently to dGTP; hence, PNP deficiency is accompanied by markedly elevated dGTP levels in cells (Cohen et al., 1978). Because dGTP concentrations are relatively low, they are best quantified by the DNA polymerase method (Sherman and Fyfe, 1989).

The impaired production of purine bases results in low serum and urine uric acid levels, an important clue in the diagnosis of PNP deficiency. Calculation of the uric acid/creatinine ratio can further enhance the sensitivity of uric acid measurement (Wortmann et al., 1979). In addition, concentrations of GTP are reduced in the erythrocytes of PNP-deficient patients to the range seen in patients with HPRT deficiency (Cohen et al., 1976; Simmonds et al., 1986).

BIOCHEMICAL BASIS AND PATHOPHYSIOLOGY

BIOCHEMISTRY OF ADA

ADA (EC 3.5.4.4) is an enzyme of the purine salvage pathway that catalyzes the irreversible deamination of adenosine and, perhaps more significantly, deoxyadenosine to inosine and deoxyinosine, respectively. The enzyme is an ubiquitous "housekeeping" enzyme that exists as a 40-kDa monomer predominantly in the cytoplasm (Daddona and Kelley, 1977; Schrader and Stacy, 1977). A small proportion of the enzyme is present on the cell surface of fibroblasts, T lymphocytes, and probably most cells, as a dimer complexed to two molecules of a combining protein, now identified as CD26 in T lymphocytes (Hirschhorn, 1975; Kameoka et al., 1993; Nishihara et al., 1973). It is hypothesized that ADA on the cell surface serves to regulate local concentrations of adenosine and thus engagement of the various cellular stimulatory and inhibitory adenosine receptors. ADA is also genetically polymorphic, with two common biochemical variants, or allozymes, segregating in the normal population (Spencer et al., 1968).

Although ADA is present in all cells, enzyme activity differs considerably in different tissues. The highest amounts are found in humans in lymphoid tissues, particularly intrathymic immature T cells, as well as brain and gastrointestinal tract, while the lowest activity is seen in erythrocytes (Hirschhorn et al., 1978). In lymphoid cells, enzyme activity declines with maturation of T cells but is higher in peripheral blood T cells

than in B cells (Tung et al., 1976). In addition to the ADA coded for on chromosome 20 and deficient in ADA deficiency (ADA1), an additional isozyme of ADA, ADA2, accounts for 1 to 2 percent of total ADA activity but deaminates adenosine only at higher-than-physiological concentrations and is not inhibited by EHNA, an inhibitor of the major ADA isozyme (Daddona, 1981). ADA2 is the product of the CECR1 (cat eye syndrome chromosome region, candidate 1) gene on chromosome 22 and belongs to novel family of ADA-related growth factors with unclear function. It has been speculated that ADA2 may be active in sites of inflammation during hypoxia and in areas where adenosine is elevated (e.g., tumor sites) (Zavialov and Engstrom, 2005).

The murine ADA enzyme has been crystallized and areas of evolutionary conservation from *E. coli* to man have been identified (Chang et al., 1991; Sharff et al., 1992; Wilson and Quiocho, 1993; Wilson et al., 1991). The enzyme is composed of a motif of eight β pleated sheets alternating with eight α helices. There are also five clearly defined additional α helices, three following the first β sheet and the fourth and fifth following the last β sheet. The β sheets form a barrel shape surrounding the catalytic site that lies at the carboxyl end of the barrel. There is a zinc ion coordinately bound at the bottom of the β barrel, under the substrate-binding site. The unexpected presence of zinc within the catalytic pocket of ADA and in other enzymes of the purine and pyrimidine salvage pathways could potentially illuminate the immunodeficiency associated with zinc deficiency. Several amino acids have been identified that are significant for binding of zinc and for catalysis. The precise mechanism of catalysis is under investigation by analysis of effect of mutations as measured by various parameters, including crystallography (Bhaumik et al., 1993; Ibrahim et al., 1995; Mohamedali et al., 1996; Sideraki et al., 1996).

Proposed Mechanisms for ADA Deficiency

Although much is known about the biochemistry of adenosine deaminase, the mechanism(s) whereby deficiency of a ubiquitous enzyme results in predominantly lymphospecific pathology remain an area of investigation (reviewed in Benveniste and Cohen, 1995; Hershfield and Mitchell, 2001; Hirschhorn, 1993; Sekhsaria et al., 1996). Based on the metabolic consequences of ADA deficiency (Fig. 14.3), several pathophysiological mechanisms have been proposed, supported by in vivo and/or in vitro findings. As listed in Table 14.4, dATP is known to be a feedback inhibitor of ribonucleotide reductase, an enzyme required for normal DNA synthesis. The observed marked elevation of dATP could thereby explain the lack of lymphocyte proliferation required for further differentiation to mature cells. The lymphospecific toxicity has been explained by in vitro studies showing that in lymphocytes, and particularly immature T cells, the pathway for phosphorylation of deoxyadenosine is more active than the reverse pathway leading to dephosphorylation; this results in intracellular trapping of the phosphorylated compound. However, while this simplistic explanation is easily remembered and therefore attractive, more recent observations suggest that preferential trapping of dATP is more closely correlated with maturity of lymphoid cells than subtype, which is further supported by the greater sensitivity of chronic lymphocytic leukemia cells and hairy-cell leukemia cells (both usually derived from B cells) to ADA inhibitors or toxic adenosine analogs (Dillman, 2004).

An additional mechanism is suggested by the finding of marked diminution of S-adenosylhomocysteine hydrolase (SAH) activity. Deoxyadenosine irreversibly inactivates the enzyme SAH hydrolase, with markedly reduced activity of this enzyme found in untreated ADA-deficient children. In vitro, this has been shown to result in accumulation of the substrate S-adenosylhomocysteine. Elevated concentrations of SAH result in feedback inhibition of the conversion of S-adenosylmethionine to S-adenosylhomocysteine and therefore prevent the transfer of methyl groups required for virtually all essential methylation reactions. The role of SAH hydrolase inactivation in the pathogenesis of the clinical findings in ADA deficiency remains to be determined. Interestingly, genetic deficiency of SAH hydrolase is not associated with

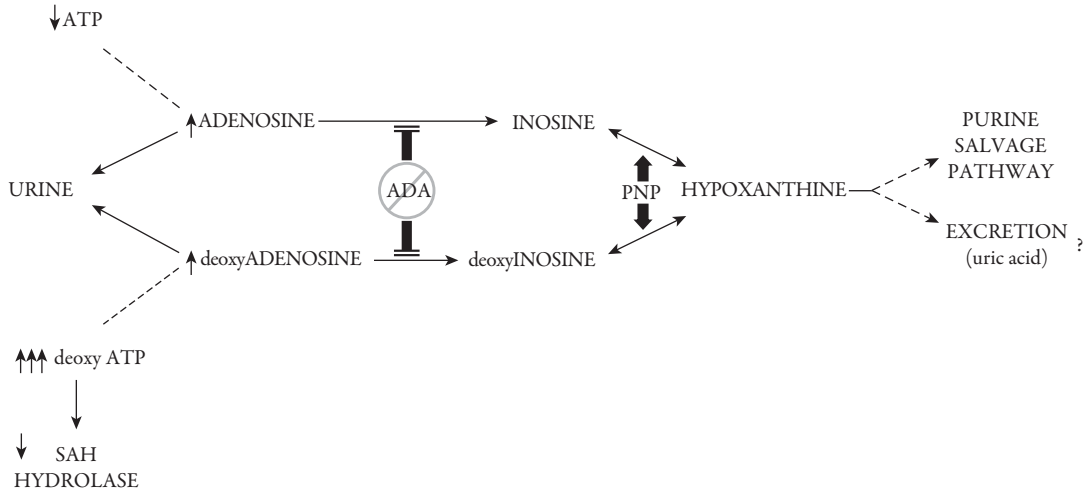

Figure 14.3 Metabolic consequences of enzyme deficiency found in ADA-deficient patients.

Table 14.4 **PROPOSED PATHOPHYSIOLOGICAL MECHANISMS IN ADA DEFICIENCY**

Elevated deoxy-ATP	**Inhibition of ribonucleotide reductase (required for DNA synthesis) and block of DNA replication**
Elevated deoxyadenosine	**Inhibition of DNA repair leading to higher number of chromosome breaks and induction of apoptosis**
Elevated adenosine	**Adenosine receptor interactions contributing to neurological/cognitive changes, sensorineural deafness, sleep abnormalities (based on pharmacology)**
Inhibition of SAH hydrolase	**Inhibition of methylation reactions (potentially contributing to neurological changes rather than immunodeficiency, based on phenotype of SAH hydrolase-deficient humans)**

immune abnormalities but does demonstrate profound psychomotor delay, hypotonia, and defects of myelination (Baric et al., 2004).

Elevation of SAH has not been demonstrated in vivo in ADA-deficient patients, and inhibition of SAH hydrolase is still found in bone-marrow-transplanted children who have normal immune function. However, administration of deoxycoformycin, a known inhibitor of ADA, to patients with leukemia was accompanied by elevations of SAH in vivo. The significance of this observation for patients with ADA deficiency is difficult to evaluate for several reasons. Deoxycoformycin also inhibits at least one other relevant enzyme, AMP deaminase, and the elevations of deoxyadenosine resulting from the death of malignant cells in the presence of ADA inhibition (and of uric acid) far exceeded that observed in any SCID patients. No explanation for why this mechanism would lead to lymphospecific toxicity has been advanced.

Elevation of cyclic AMP in lymphocytes, known to inhibit lymphocyte function, has also been demonstrated in vitro but not in vivo. A proposed mechanism, inhibition of pyrimidine biosynthesis by elevation of ATP, can no longer be supported because in fact, decreased rather than increased ATP is found in SCID patients with the most marked elevations of dATP (the original report of elevated ATP used an assay that measured dATP as well as ATP). Additional pathophysiological mechanisms have been proposed based on in vitro observations (e.g., activation of ATP catabolism by deoxyadenosine and interference with terminal transferase by dATP); however, they have not yet found confirmation from in vivo data. In summary, while the bulk of in vivo and in vitro data support dATP as an important toxic metabolite, this does not explain several in vitro observations. It is likely that additional mechanisms are operative.

It is also clear that all pathophysiological mechanisms in ADA deficiency result from the presence of increased concentrations of substrates of ADA. These may be particularly elevated in the thymus, within which lymphoid cells differentiate and normally undergo apoptosis, and in infected or inflamed tissues, where mature lymphoid cells are engaged in an immune response. Deoxyadenosine has been reported to result in chromosome breakage and to induce apoptosis in relatively immature thymic lymphocytes by a pathway found after DNA breakage, requiring expression of p53 and reversible by overexpression of Bcl-1. These studies correlate with the high expression of p53 and low expression of Bcl-1 in immature intrathymic lymphocytes and suggest that a major block in lymphocyte differentiation resulting from ADA deficiency is intrathymic (Benveniste and Cohen, 1995). However, GM-CSF-stimulated recovery of $CD34^+$ hematopoietic progenitors is lower in ADA-deficient patients than in normals, suggesting that there is also a block at an earlier stage (Sekhsaria et al., 1996).

In addition to affecting development of T lymphocytes, ADA deficiency results in T-cell dysfunction. Primary CD4 T-cell lines from ADA-SCID patients have defective proliferative responses to TcR stimulation, associated with impaired downstream signaling (ZAP-70 phosphorylation, ERK activation, and calcium flux), that may contribute to the immunodeficiency in affected patients (Cassani et al., 2008). In addition, high adenosine concentrations interfere with regulatory T cell function (Sauer et al., 2012a), which may contribute to the susceptibility to autoimmune complications that are not uncommon in ADA-deficient patients.

Molecular Biology of PNP

Native human PNP is a trimer of 3 identical 32,153-Da subunits, each with a substrate-binding site. PNP has been isolated from a wide variety of species, including bacteria (e.g., *E. coli, Haemophilus influenzae*), protozoa (e.g., *Plasmodium falciparum*), rodents (e.g., mice and rats), and mammals, including cows and chimpanzees. A high degree of homology exists between these PNP enzymes, with human and bovine and the human and murine PNPs demonstrating 87 percent and >84 percent homology, respectively (Li et al., 2008; Nelson et al., 1992). PNP from *E. coli*, Schistosoma, bovine, human, and other species has been crystallized with various compounds and analogs in the search for specific PNP inhibitors (Bennett et al., 2003; Canduri et al., 2005; Caceres et al., 2010). Studies that revealed the active site of the PNP enzymes enabled designing of selective PNP inhibitors (Ealick et al., 1991, 1993). Notably, Immucillin H, commercially known as Forodesine, which abrogates PNP function in human T cells, is being used in clinical trials for T-cell lymphomas and leukemia (Alonso et al., 2009; Gandhi et al., 2005). Similarly, specific PNP inhibitors might be beneficial for treatment of T-cell-mediated autoimmune diseases and allograft rejection.

PNP from all species tested can metabolize guanosine; however, the enzymes differ in their action on other purine analogs. This led several groups to investigate the possibility of delivering *E. coli* PNP into human tumor cells to selectively induce susceptibility of these cells to purine metabolites. A phase I study is investigating the expression of *E. coli* PNP in prostate cancer cells followed by treatment with a pro-drug, fludarabine, that is converted by the *E. coli* PNP, but not by the human PNP, into highly cytotoxic 2-fluoroadenine (Hebrard et al., 2009).

PNP is a member of the nucleoside phosphorylase-I (NP-I) family. The NP-I family includes enzymes that share a common single-domain subunit, with either a trimeric or a hexameric quaternary structure that accepts both purine and pyrimidine nucleoside substrates. Despite species-dependent differences in the quaternary structures of members of the NP-I family, the subunit fold is highly conserved with a central β sheet that forms a distorted β barrel, surrounded by several α helices. The active site in members of the NP-I family consists of adjacent phosphate- and nucleoside-binding pockets. Residues from the central β sheet, interconnecting loops, and an adjacent subunit form the binding site. The central β-sheet structural motif, where the nucleoside and phosphate bind at the C-terminal end of the strands, is completely conserved. This suggests that the β-sheet structural motif is important for nucleoside and phosphate binding and catalyzing phosphorolysis (Pugmire and Ealick, 2002).

PNP has been identified in the cytoplasm of cells (Rubio and Berne, 1980; Toro and Grunebaum, 2006) and also found associated with centrioles and basal bodies (Oliver et al., 1981). Recent studies also suggest that the enzymes involved in purine synthesis are co-localized in a cytoplasmic complex named the purinosome (An et al., 2008). However, it is still unclear whether purine degradation enzymes, such as PNP, are organized in a similar manner. In contrast to ADA, PNP has not been detected on the surface of cells.

PNP is considered a ubiquitous "housekeeping" enzyme, but its expression and activity differ considerably in different cells. The highest activity of PNP is in peripheral blood granulocyte and lymphoid cells. Among human T-lineage cells, PNP activity in peripheral T lymphocytes (63 + 4 nmol/minute/10^8 cells) is significantly higher than in thymocytes (18 + 2 nmol/minute/10^8 cells) or leukemic T-ALL cells (37 + 3 nmol/minute/10^8 cells) (van de Griend et al., 1983). In human gastrointestinal tissue, PNP was detected in the duodenum but not in the tongue or esophagus (Witte et al., 1991). In mice, the highest PNP activity was found in the small intestine, followed by the tongue and esophagus. Northern blot analysis in these mice also demonstrated PNP mRNA in the liver, lungs, and kidney (Mohamedali et al., 1993). In adult neuronal tissue, PNP is expressed in white and gray brain astrocytes and microglia but not in neurons (Castellano et al., 1990).

Proposed Mechanisms for PNP Deficiency

Several mechanisms have been proposed to explain the effects of PNP deficiency on cells in general and on T-lineage cells in particular. Most researchers have focused on the intracellular accumulation of PNP nucleoside substrates and their metabolic products (Hershfield and Mitchell, 2001). Among the four PNP purine substrates that accumulate in PNP deficiency, only deoxyguanosine has an alternative metabolic fate, as it can be phosphorylated by the mitochondrial deoxyguanosine kinase (dGK) or the nuclear deoxycytidine kinase (dCK) to deoxyguanosine monophosphate (dGMP), as described in Figure 14.4. dGMP can undergo further phosphorylation to deoxyguanosine diphosphate (dGDP) and subsequently to deoxyguanosine triphosphate (dGTP), which are trapped inside the cells.

Evidence for the central role of deoxyguanosine in mediating the effects of PNP deficiency includes the unique ability of deoxyguanosine, but not other PNP substrates, to inhibit the growth of T cells. This inhibition was associated with an increased intracellular concentration of dGTP (Gudas et al., 1978; Ullman et al., 1979), supporting the hypothesis that the toxicity was caused by phosphorylation of deoxyguanosine to dGTP. Moreover, the inhibitory effect of deoxyguanosine was abolished by disruption of its delivery into cells or by inactivation of dGK or dCK, which prevents the formation of dGTP (Ullman et al., 1979). Indeed, dGTP concentrations

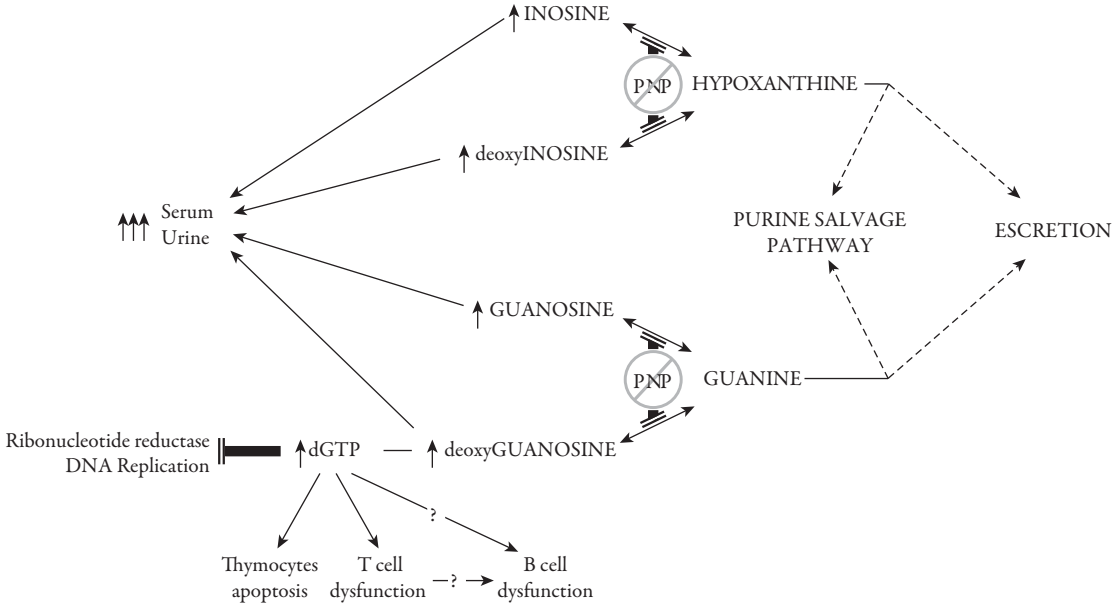

Figure 14.4 Metabolic and proposed cellular consequences of PNP deficiency.

are markedly increased in the cells of PNP-deficient patients (Cohen et al., 1978), in cells with chemically inhibited PNP activity (Bantia et al., 2003; Gandhi et al., 2005), and in cells from PNP-deficient mice (Arpaia et al., 2000).

Maintenance of low and balanced concentrations of intracellular deoxynucleoside triphosphates, including dGTP, is essential for the integrity of DNA synthesis and repair. dGTP binds and inhibits the function of ribonucleoside reductase, an enzyme responsible for the de novo synthesis of deoxyribonucleotides from ADP, GDP, UDP, and CDP. Inactivation of ribonucleoside reductase was also found to prevent the inhibitory effects of deoxyguanosine (Ullman et al., 1979), which led to the model that increased dGTP, secondary to accumulation of deoxyguanosine, may also affect cells by interfering with the synthesis of other deoxyribonucleotides.

Both an enhanced ability to accumulate dGTP and increased sensitivity to dGTP have been suggested as causes for the preferential T-lineage abnormalities in PNP deficiency. Human thymocytes were found to have increased dGK activity and increased dGTP concentrations compared to peripheral T and B lymphocytes (Cohen et al., 1980). Moreover, the mitochondrial localization of dGK and the increased dGTP concentrations in the mitochondria of thymocytes from PNP-deficient mice suggested that mitochondrial DNA maintenance might be particularly affected by PNP deficiency (Arpaia et al., 2000).

Support for this hypothesis was provided by accumulation of dGTP in the mitochondria of PNP-expressing thymocytes incubated with deoxyguanosine in the presence of a PNP inhibitor (Arpaia et al., 2000). Moreover, dissipation of mitochondrial membrane potential in these cells was resistant to caspase inhibition, indicating that the apoptotic signals initiated by deoxyguanosine originated within the mitochondria. Interestingly, Northern blotting demonstrated increased mRNA for dCK, but not dGK, in normal human thymus (Johansson and Karlsson, 1996), suggesting that dCK expression may also have a significant role in the increased sensitivity of human thymocytes to PNP deficiency.

A second explanation for the preferential damage to thymocytes in PNP deficiency stems from the increased propensity for apoptosis of immature thymocytes, which normally downregulate anti-apoptotic molecules such as Bcl-2, during selection in the thymus. Studies in the murine models of PNP deficiency demonstrated that thymocyte differentiation is severely affected at the $CD4^+$ $CD8^+$ double-positive stage, with increased apoptosis of double-positive thymocytes (Arpaia et al., 2000; Snyder et al., 1997). Furthermore, deoxyguanosine was found to induce apoptosis of double-positive thymocytes isolated from either normal mice treated with a PNP inhibitor or PNP-deficient mice (Arpaia et al., 2000; Papinazath et al., 2011). In addition, Bcl-2 overexpression completely protects against deoxyguanosine-induced apoptosis in murine thymocytes treated with a PNP inhibitor (Arpaia et al., 2000). Further exacerbation of thymocyte damage may result from locally increased toxic purine metabolites released from dying thymocytes.

Alterations in intracellular purine dNTP pools might affect V(D)J recombination in ADA- and PNP-deficient thymocytes. Cell lines representing various T-lineage stages, treated with deoxyguanosine, demonstrated that an increase in dGTP concentration was associated with a two-fold reduction of signal-joint recombination in immature T cells, although the ratio of G + C/A + T *N*-region composition was not significantly altered (Gangi-Peterson et al., 1999). Thus, the abnormal accumulation of dGTP in maturing thymocytes may affect V(D)J recombination efficacy or interfere with DNA integrity and result in enhanced cell death in these cells that are already prone to undergo apoptosis.

The reduced response of T cells from PNP-deficient patients to mitogens and the increased sensitivity of such cells to ionizing irradiation (Dror et al., 2004) suggests that in addition to causing abnormal thymocyte development, PNP deficiency may directly affect peripheral T cells. Incubation of PNP-proficient cells with deoxyguanosine and PNP inhibitor, to simulate PNP deficiency conditions, causes increased apoptosis of T-cell lines and stimulated T cells but has practically no effect on unstimulated T cells (Carson et al., 1982; Wilson et al., 1979). Lymphocytes from patients with T-cell malignancies treated in vitro or in vivo with chemical PNP inhibitors demonstrate increased dGTP accumulation and reduced proliferation (Bantia et al., 2003; Conry et al., 1998; Gandhi et al., 2005). The differences in susceptibility of thymocytes, activated lymphocytes, and nonstimulated lymphocytes to abnormal purine metabolism could be attributed to the lack of dCK expression and markedly reduced dGK expression in nonstimulated T lymphocytes (Johansson and Karlsson, 1996). These observations and our recent findings of increased thymidine incorporation into freshly isolated T lymphocytes from PNP-deficient mice (Yu et al., 2009), suggests that T-lymphocyte abnormalities in PNP deficiency may result from in vivo activation of peripheral lymphocytes, possibly secondary to unrecognized infections or uncontrolled systemic inflammation. Alternatively, PNP deficiency might disrupt lymphocyte intracellular signaling, as we have shown abnormal cytokine production by PNP-deficient lymphocytes (Arpaia et al., 2000; Dror et al., 2004; Toro and Grunebaum, 2006).

The effects of abnormal PNP activity on the brain and other organs have not been elucidated. GTP depletion due to inability to produce guanine or secondary to overconsumption of phosphoribosyl pyrophosphate interferes with de novo purine synthesis (Barankiewicz et al., 1982) and has been suggested as a cause for the neurological symptoms in PNP-deficient patients, similar to the effects of HPRT deficiency (Simmonds et al., 1987). However, direct proof in support of this hypothesis has not been provided.

Thus, characterization of the deficits associated with PNP deficiency, identification of the precise tissues and differentiation stages involved, and availability of tissues and animal models that recapitulate the symptoms seen in PNP-deficient patients are required to better understand the pathogenesis of the disease.

MOLECULAR BIOLOGY

MOLECULAR ORGANIZATION OF THE ADA GENE

The human gene for ADA (*ADA*) is localized on the long arm of chromosome 20 at 20q12-q13.1. Both the cDNA and the genomic DNA have been isolated and analyzed (Aronow et al., 1995; Daddona et al., 1984; Valerio et al., 1984, 1985; Wiginton et al., 1984, 1986). The cDNA for human ADA is at least 1.5 kb in length and contains a coding region of 1,089 nucleotides, including the initiation methionine codon ATG, predicting a polypeptide of 363 amino acids with a calculated M.W. of 40,638 Daltons. The ADA cDNA is contained in 12 exons within 32,040 nucleotides of genomic DNA, from the major cap site at position 95 from the ATG to the polyadenylation site. The first 230 bp upstream of the coding region are sufficient to promote expression of the ADA cDNA, as tested by DNA-mediated gene transfer of a minigene construct. The promoter is of the GC-rich type with binding sites for Sp1, now recognized in many housekeeping genes, and lacks obvious CAAT or TATA boxes. Additional regulatory sequences are present in the large (15 kb) first intron, including an enhancer that is responsible for high expression in T cells. Studies with transgenic mice indicate that sequences controlling differences in tissue expression are contained in the first intron, with enhancer elements and elements directing high expression in T lymphocytes as well as an element responsive to the transcription activator cMyb (Aronow et al., 1995). The seventh intron is very small and appears to be unspliced in a small, but detectable, portion of ADA mRNA in normals. The gene is very rich in repetitive sequences of the Alu type; 23 copies of Alu account for 18 of the total sequence. Seventeen Alu repeats are in the first two introns, with three additional Alu repeats in the 5 upstream region. Alu repeats throughout the genome are known to be sites for gene deletions and duplications resulting from misalignment when homologous chromosomes pair during meiosis followed by unequal recombination. Alu repeats have been shown to be involved in causing deletions in the ADA gene (see below).

MUTATION ANALYSIS OF ADA

The types and variety of mutations are not the same in all genetic diseases (see Chapter 2). The predominant mutations seen can vary at different genetic loci or with differing phenotypes at the same locus. Moreover, at different genetic loci there can be many different mutations or a few major mutations causing the majority of the disease cases. Finally, even when there are multiple different mutations, a particular population or ethnic group may demonstrate only a few mutations accounting for all patients with disease in that group.

Mutations in Immunodeficient Patients

In ADA deficiency there are multiple different mutations, but some are more common than others and are found in unrelated individuals (Fig. 14.5). Several of the more common mutations share ancestral origins, while others represent independent recurrences of mutation at "hot spots" within the ADA gene. In the initial cases reported, there was a predominance of single base-pair changes resulting in amino acid substitutions, or missense mutations at mutational hot spots. In later-onset patients, there appears to be an increased incidence of splice-site mutations. There is also an increasing number of recognized small deletions and insertions, as the methods of ADA patient diagnosis have turned to examination of genomic DNA. Some ethnic concentrations of particular mutations appear to be emerging.

Over 50 deleterious mutations have been identified in ADA-deficient immunodeficient patients. An additional 11 "partial" mutations have been identified. Table 14.5 lists published mutations, while Table 14.6 indicates mutations found in multiple unrelated patients by the Hirschhorn laboratory. Additional mutations have been identified but are not as yet published from this laboratory. Approximately two thirds of the deleterious mutations have been reported

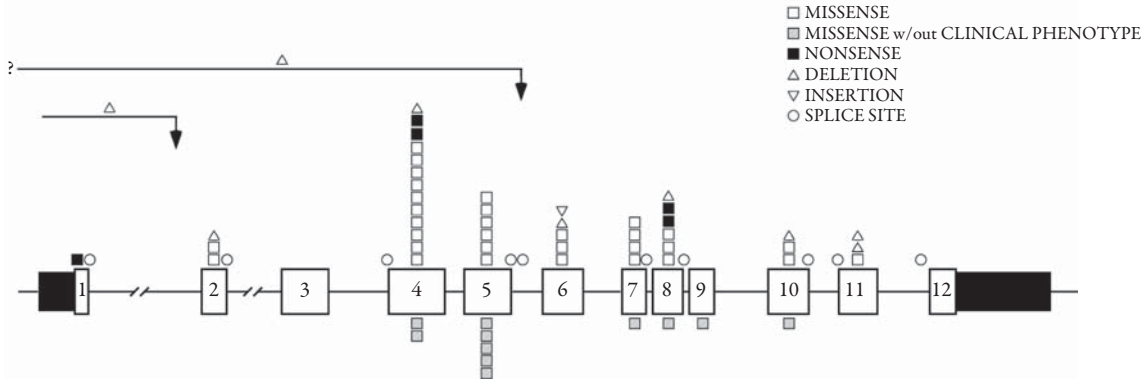

Figure 14.5 Mutations at the ADA locus.

Table 14.5 MUTATIONS AT THE ADA LOCUS

EFFECT	SITE	SEQUENCE VARIATION	REFERENCE
A. Missense Variants			
1. In immunodeficient patients			
p.His15Asp	exon 2	c.43C>T	Santisteban et al., 1995a
p.Gly20Arg	exon 2	c.58G>A	Yang et al., 1994
p.Gly74Cys	exon 4	c.220G>T	Arrendondo-Vega et al., 1998
p.Gly74Val	exon 4	c.221G>T	Bollinger et al., 1996
p.Gly74Asp	exon 4	c.221G>A	Ariga et al., 2001a
p.Ala83Asp	exon 4	c.248C>A	Santisteban et al., 1995a
p.Tyr97Cys	exon 4	c.290A>G	Jiang et al., 1997
p.Arg101Trp	exon 4	c.301C>T	Akeson et al., 1988
p.Arg101Gln	exon 4	c.302G>A	Bonthron et al., 1985
p.Arg101Leu	exon 4	c.302G>T	Santisteban et al., 1993
p.Pro104Leu	exon 4	c.311C>T	Atasoy et al., 1993
p.Leu107Pro	exon 4	c.320T>C	Hirschhorn et al., 1990
p.Pro126Gln	exon 5	c.377C>T	Ozsahin et al., 1997
p.Val129Met	exon 5	c.385G>A	Arrendondo-Vega et al., 1998
p.Gly140Glu	exon 5	c.419G>A	Arrendondo-Vega et al., 1998
p.Arg149Trp	exon 5	c.445C>T	Arrendondo-Vega et al., 1998
p.Arg156Cys	exon 5	c.466C>T	Hirschhorn, 1992
p.Arg156His	exon 5	c.467G>A	Santisteban et al., 1993
p.Arg156Leu	exon 5	c.467G>T	Artac et al., 2009
p.Val177Met	exon 6	c.529G>A	Santisteban et al., 1993
p.Ala179Asp	exon 6	c.536C>A	Santisteban et al., 1995a
p.Gln199Pro	exon 6	c.596A>C	Arrendondo-Vega et al., 1998
p.Arg211Cys[a]	exon 7	c.631C>T	Hirschhorn et al., 1990
p.Arg211His	exon 7	c.632G>A	Akeson et al., 1988
p.Gly216Arg	exon 7	c.646G>A	Hirschhorn et al., 1991
p.Glu217Lys	exon 7	c.649G>A	Hirschhorn et al., 1992
p.Arg235Trp	exon 8	c.703C>T	R. Hirschhorn, unpublished*
p.Arg235Gln	exon 8	c.704G>A	Ariga et al., 2001b
p.Arg253Pro	exon 8	c.758G>C	Hirschhorn et al., 1993b
p.Ser291Leu	exon 10	c.872C>T	Hirschhorn, 1992
p.Leu304Arg	exon 10	c.912T>G	Valerio et al., 1986
p.Ala329Val	exon 11	c.986C>T	Akeson et al., 1988
2. In clinically normal individuals with diminished red blood cell ADA but presence of substantial ADA in other cells ("partial ADA deficiency")			
p.Arg76Trp	exon 4	c.226C>T	Hirschhorn et al., 1990
p.Leu106Val	exon 4	c.316C>G	Jiang et al., 1997
p.Arg142Gln	exon 5	c.425G>A	Santisteban et al., 1995b
p.Arg149Gln	exon 5	c.446G>A	Hirschhorn et al., 1990
p.Leu152Met	exon 5	c.454C>A	Hirschhorn et al., 1997
p.Ala215Thr	exon 7	c.643G>A	Hirschhorn et al., 1990; Ozsahin et al., 1997
p.Thr233Ile	exon 8	c.698C>T	Hirschhorn et al., 1997
p.Gly239Ser	exon 8	c.715G>A	Ariga et al., 2001a
p.Pro274Leu	exon 9	c.821C>T	Hirschhorn et al., 1990

Table 14.5 (CONTINUED)

EFFECT	SITE	SEQUENCE VARIATION	REFERENCE
p.Pro297Gln	exon 10	c.891C>A	Hirschhorn et al., 1989
p.Met310Thr	exon 10	c.930T>C	Ariga et al., 2001a
3. In normals (common SNPs)			
p.Asp8Asn	exon 1	c.22G>A	Hirschhorn et al., 1994a
p.Lys80Arg	exon 4	c.239A>G	Valerio et al., 1986
B. Nonsense Mutations			
p.Gln3X	exon 1	c.7C>T	Santisteban et al., 1995a
p.Glu99X	exon 4	c.295G>T	Honig et al., 2007
p.Gln119X	exon 4	c.355C>T	Ariga et al., 2001b
p.Arg142X	exon 5	c.424C>T	Santisteban et al., 1995a
p.Gln246X	exon 8	c.736C>T	Honig et al., 2007
p.Gln254X	exon 8	c.760C>T	Hirschhorn, 1993
C. Splice-Site Mutations			
unstable mRNA	IVS 1	c.33+1G>C	Hirschhorn et al., 1994b
unstable mRNA (deletes exon 2; + use of cryptic site)	IVS 2	c.95+1G>A	Arredondo-Vega et al., 1994 Onodera et al., 1998
deletes exon 4	IVS 3	c.218+2A>G	Akeson et al., 1987, 1988
unstable mRNA (deletes exon 5)	IVS 5	c.478+1G>A	Santisteban et al., 1995a
deletes exon 5	IVS 5	c.478+6T>A	Santisteban et al., 1993
unstable mRNA (deletes exon 7)	IVS 7	c.678+1G>A	Kawamoto et al., 1993
unstable mRNA (deletes exon 9)	IVS 8	c.781-4_781-3insTGGAAGAG + c.781_782insTCTGG	Arredondo-Vega et al., 1994
unstable mRNA (+ use of cryptic splice)	IVS 10	c.975+1G>A	Santisteban et al., 1993
+32 bp & 100 aa	IVS 10	c.976-34G>A	Santisteban et al., 1993
+13 bp & 43 aa	IVS 11	c.1079-15T>A	Arredondo-Vega et al., 2001
D. Deletions/Insertions			
no mRNA	exon 1	Deletion promoter & exon 1 (Alu-Alu recombination)	Markert et al., 1988 Berkvens et al., 1990
p.Pro55_Thr57del	exon 2	c.70_78delCCTGAAACC	Honig et al., 2007
p.Pro104fsX132	exon 4	c.314delA	Ariga et al., 2001b
no mRNA	exon 5	Deletion promoter & exons 1_5 + c.367delG	Hirschhorn et al., 1992 Arrendondo-Vega et al., 1998
no mRNA	exon 6 exon 6	c.539_540delTT Stop (TGA). codon 185 c.577_578insC Premature stop	Kawamoto et al., 1993 Unpublished
p.Arg235fsX310	exon 8	c.705delG	Liu et al., 2009
unstable mRNA	exon 10 exon 11 exon 11	c.955_559delGAAGA Stop codon 320 c.1009_1011delGAA c.1019_1020delAG Stop codon 348	Hirschhorn et al., 1993a Gossage et al., 1993 Arrendondo-Vega et al., 1998 Santisteban et al., 1993

NCBI Reference Sequence: NM_000022.2. *Unpublished data are from Hirschhorn, Yang, and Jiang.

Table 14.6 ADA MUTATIONS FOUND IN MULTIPLE RELATED AND UNRELATED PATIENTS

MUTATION	NUMBER OF PATIENTS	INDEPENDENT CHROMOSOMES
c.986C>T; p.Ala329Val	9	12
del promoter-exon 1	7	9
c.646G>A; p.Gly216Arg	6	7
c.955_559delGAAGA	4	5
c.467G>A; p.Arg156His	4	4
c.478+1G>A	4	4
c.872C>T; p.Ser291Leu	4	4
c.632G>A; p.Arg211His	4	5
c.424C>T; p.Arg142X	3	6
c.58G>A; Gly20Arg	2	3
c.221G>T; p.Gly74Val	3	3
c.631C>T; p.Arg211Cys	2	2
Total	52	64/104

by the Hershfield group (Duke University Medical Center). With respect to type of mutation, the majority of the clearly deleterious mutations (31) are missense mutations, followed by 11 deletions or insertions, 10 splice-site mutations, and 6 nonsense mutations. The missense mutations appear to be concentrated in exons 4, 5, and 7, regions known to encode amino acids involved in binding of substrate or significant for the catalytic mechanism. Despite the multiplicity of mutations, 12 mutations accounted for almost two thirds of mutant chromosomes in 52 patients studied in the Hirschhorn laboratory (Table 14.6). As a result, the majority of patients carry 1 of these 12 mutations on at least one chromosome. Additionally, the leucine-to-proline mutation at amino acid 107 (p.Leu107Pro) and neighboring proline-to-leucine at position 104 (p.Pro104Leu) have also been found in more than one patient, as has the Gln3stop mutation that has been found in several Somali patients (Sanchez et al., 2007; Santisteban et al., 1995b). As more patients from different areas are studied, this distribution is likely to change. Over two thirds of patients are heteroallelic for two different mutations, with the remainder homozygous for the same mutation. Homozygosity is typical of rare autosomal recessive diseases, for which consanguinity is an important risk factor. In the case of ADA deficiency, many patients with homozygous mutations are from populations with a high frequency of inbreeding, including the American Amish community in Pennsylvania (p.Gly216Arg), Swiss and French Canadians (deletion of five nucleotides of exon10), inhabitants of Newfoundland (p.Gly20Arg), Afro-Americans (p.Ala329Val), Canadian Mennonites (p.Arg142X), and Somali patients (p.Gln3X).

The presence of the same mutation in unrelated individuals can result from descent from an unidentified common ancestor or from independent recurrence, usually at a site that is a hot spot for mutation. Descent from a common ancestor can be suspected if there is a common ethnic background or if both patients are also identical for other uncommon DNA polymorphic markers within the ADA gene, suggesting an ancestral haplotype (Chapter 2). Conversely, independent recurrence is indicated by differences in alleles at closely associated polymorphic DNA markers. Hot spots for independent recurrence of mutations include CpG dinucleotides (representing 30 of all human disease-related mutations), highly repetitive sequences (such as Alu sequences), and small repeat sequences that have been identified empirically. Over half of the missense mutations described to date in ADA-deficient patients are at CpG dinucleotides, a frequency much greater than that reported in other diseases and setting the stage for independent recurrence. As an example, the p.Ala329Val mutation, the single most commonly occurring ADA mutation, is at a CpG hot spot, in which the C residue of the GCG codon for alanine is changed to T. The mutation is found on two different haplotypes, one in Afro-Americans and the second in Caucasians, consistent with independent recurrence in each of these groups at some time in the past.

One of the two reported large deletions, including the gene promoter region and exon 1, is relatively common and also appears to have arisen independently in different patients (Table 14.6) (Jiang et al., 1997; Shovlin et al., 1994). The deletion of the promoter and exon 1 occurs through homologous recombination between two Alu repeats surrounding the promoter and first exon. Such a deletion has been reported in three patients, but with different sequences at the junctions of the deletion, indicating independent recurrence. The presence of this deletion can be easily missed unless tested for specifically by currently used methods of mutation detection, including screening methods such as SSCP or analysis of cDNA or of PCR-amplified genomic DNA. In a study of 29 patients, we have found this mutation in four patients in whom we had been initially unable to identify the mutation on the second allele, indicating that this deletion is a relatively common mutation (R. Hirschhorn, unpublished data). By contrast with the independent recurrence of the promoter/exon 1 deletion, a 5-bp deletion in exon 10 has been identified in at least four unrelated patients but appears to have derived from a common ancestor in these apparently unrelated individuals. In all four patients, the deletion is found on an extremely rare chromosomal background defined by the presence of two rare nondeleterious missense mutations (normal variants) and, in the three patients studied more fully, of a rare combination of RFLPs (haplotype V) (Tzall et al., 1989). Nonetheless, the deletion occurs at a site empirically identified with an increased frequency of small deletions and has homology with a site for topoisomerase; the deletion of five bases would fit with loss of a half-turn of the helix due to breaks associated with topoisomerase. It is therefore possible for this mutation to recur independently.

A number of splice-site mutations have been reported in individuals with later onset of disease. It can be expected that more nonsense mutations, small deletions, small insertions, and splice-site mutations (all of which can result in unstable mRNA) will be found as the predominant method of analysis changes from examination of cDNA to sequence analysis of genomic DNA.

The cataloguing of mutations in ADA-deficient patients suggests that diagnosis of the specific mutation(s) present in a newly diagnosed patient may be aided by considering the frequency of different mutations and the ethnic distribution. For example, population studies in Somalis have shown that a common haplotype carrying the Gln3Stop mutation had a carrier frequency of 2.4 percent, indicating that ~1 of 5,000 to 10,000 Somali children may be expected to be born with ADA deficiency due to this mutation (Sanchez et al., 2007).

Mutations in Partially Deficient Individuals

In addition to mutations found in immunodeficient patients, 11 different missense mutations retaining differing amounts of easily detectable enzyme activity in nonerythroid cells have been identified in the course of screening erythrocytes from clinically normal individuals. These individuals were categorized as having partial ADA deficiency, based on the absence of red cell ADA but the presence of residual (5 to 80 percent of normal) ADA in nonerythroid cells and identification either by screening of normals or as an incidental finding in apparently healthy relatives of ADA-deficient subjects. Several ADA variants have been found in multiple individuals, many of whom derive from the same geographical area in the Caribbean or share African descent (Hirschhorn et al., 1990). One of these partial mutations, p.Arg211Cys, is listed with mutations in immunodeficient patients (Table 14.5). Originally identified through population screening of newborns, hetero-allelic with a null mutant allele, this mutation has now been identified in siblings with adult-onset immunodeficiency whose mutation on their opposite ADA allele is the del promoter-exon 1 (Table 14.4) (Daddona et al., 1983; Hirschhorn et al., 1990, 1997; Shovlin et al., 1993). It is possible that the original unaffected child identified through screening with the p.Arg211Cys mutation will develop immunodeficiency. The remaining two missense mutations were identified in healthy relatives of ADA-deficient patients (p.Gly 239Ser and p.Met310Thre) as was a second instance of the p.Ala215Thr mutation. Additional partial mutations that express very low, but easily detectable, ADA activity (p.Leu152Met), when hetero-allelic with null mutations, may be found in patients with later-onset immunodeficiency. It is noteworthy that the majority of patients are of "African" descent (including a !Kung tribesman), and a selective advantage for malaria is possible.

EFFECT OF DIFFERENT MUTATIONS ON RESIDUAL ADA ACTIVITY

As is most readily apparent from the study of mutations found in partially ADA-deficient immunocompetent individuals, mutations may not totally abolish enzyme activity (Table 14.7). This is also true for mutations found in immunodeficient individuals. In published and unpublished studies, we have compared the ability of over 20 different missense mutations to code for ADA with residual activity in vitro. The mutations fall into four different groups. Approximately half of the mutations found in immunodeficient patients do not express detectable enzyme activity. The remainder of the missense mutations in immunodeficient patients express detectable residual ADA activity, generally 0.5 to 2 percent of normal. (An exception is the mutation p.Arg211Cys, found in adult-onset immunodeficiency, which expresses somewhat more than 2 percent of normal activity.) A borderline group of ADA mutations expressing 3 to 6 percent of normal activity contains the above mutation p.Arg211Cys, originally found in heterozygosity in a child ascertained by newborn screening, but more recently found hetero-allelic with a large deletion in adult-onset immunodeficiency in siblings. Also in this category is the p.Leu152Met mutation present in homozygosity in a child ascertained by newborn screening who was currently healthy at 12 years of age but had a prior sibling who died in infancy with an infectious disease (Hirschhorn et al., 1997). Finally, mutations expressing 10 to 80 percent of normal activity are found by screening of normal individuals. These variants may confer instability upon the mutant enzyme or have a direct effect upon the binding of substrate, disruption of the zinc-interacting residues, or altering residues governing the catalytic mechanism. Although some of these effects can be hypothesized based on the crystalline structure of the murine ADA molecule, definitive conclusions await direct examination of mutant enzymes.

Table 14.7 **RELATIVE RESIDUAL ADA ACTIVITY OF MUTANT ENZYMES**

ENZYME ACTIVITY (MUTATIONS LISTED IN ORDER OF DECREASING ACTIVITY)			
NOT DETECTABLE	0.5–2%	2.5–6%	10–80%
p.Gly20Arg	p.Arg156His	p.Arg211Cys	p.Arg149Gln
p.Pro104Leu	p.Arg253Pro	p.Leu152Met	p.Pro297Gln
p.Gly216Arg	p.Arg156Cys		p.Thr233Ile
p.Glu217Lys	p.Ala329Val		p.Arg 76Trp
p.Arg235Trp	p.Gly74Val		p.Pro274Leu
p.Ser291Leu	p.Arg211His		p.Ala215Thr
p.Arg101Trp	p.Leu107Pro		
	p.Leu304Arg		

Analysis of activity of different mutations has also been measured by expression in bacteria (*E. coli*) that are genetically devoid of endogenous ADA activity (Arredondo-Vega et al., 1998; Hershfield and Mitchell, 2001). Based upon the activity expressed in *E. coli*, various mutations were divided into four different groups and appeared to correlate well with clinical and biochemical phenotype. Fourteen of the 29 mutations tested in *E. coli* had also been tested for expression in mammalian cells (Table 14.7, Hirschhorn and Yang, unpublished). Mutations with the highest activity (class IV and class III) were those seen in partially deficient patients.

GENOTYPE–PHENOTYPE CORRELATIONS

Correlations of phenotype with genotype are being examined for many inherited disorders, with the increased definition of mutations at multiple different disease loci. However, other genetic and nongenetic factors can greatly modify the disease phenotype, despite the presence of identical mutations in different patients. Phenotypic differences between siblings carrying the same mutation(s) could be due to modifying genes unlinked to the primary disease locus. Differences between unrelated individuals carrying the same mutation have been attributed to differences in chromosomal background within the gene. Environmental factors can also modify genetic disease phenotypes to a greater or lesser extent, depending upon the particular disease. Lastly, a milder phenotype than predicted by the specific mutation can result from somatic mosaicism.

In an autosomal recessive disorder due to defects in a monomeric enzyme, such as ADA, it would be expected that the presence of a null allele with a mutant allele expressing residual ADA activity would result in a mild phenotype. Homozygosity for two such mutant alleles could theoretically result in an even milder phenotype. The expression of residual ADA could result from a missense mutation that does not totally abolish enzyme activity or of a mutation at a splice site that demonstrates "leakiness" (that is, allows some degree of normal splicing). Critical evaluation of phenotype versus genotype would be best carried out by comparing unrelated patients with identical homozygous mutations to siblings carrying the same mutations. However, for a disorder as rare as ADA deficiency, definitive correlations may not be possible. Moreover, environment, particularly exposure to and incidence of infections, could play a major modifying role. As discussed more fully below, somatic mosaicism, due to de novo mutations during embryogenesis or to reversion to normal of one of the inherited mutations, can dramatically modify the phenotype.

Despite these caveats, correlations between specific mutations, metabolite concentrations, residual ADA activity, age of onset, and severity of disease appear to be present in ADA deficiency. Several mutations in homozygosity or quasi-homozygosity have been recognized in neonatal-onset patients that are consistent with the phenotype. These include a p.Gly20Arg, p.Glu217Lys, and p.Gly216Arg (hetero-allelic with a large deletion including the promoter through exon 5), all of which missense mutations are at critical areas of the molecule. The deletion of five nucleotides in exon 10 and possibly the p.Arg142X mutations may also be associated with neonatal-onset SCID. As noted above, several splice-site mutations and the p.Arg211Cys mutation expressing some activity appear to be associated with later onset and milder perturbations of immune function and of metabolites. While relative concordance between siblings (modified by early institution of supportive therapy) has been noted, marked discordance between one set of siblings has also been reported, suggesting the existence of modifying genes or other factors (Arredondo-Vega et al., 1994). The discordant siblings were heterozygous for two different splice mutations. One allele had in intron 2, at the invariant position IVS2 1, a guanine changed to an adenosine nucleotide. The other allele bore a complex 17-bp rearrangement in intron 8 of the 3′ splice site of IVS8; this mutation involved insertion of the purine-rich antisense strand of the PY tract between the conserved CAG and the normal PY tract of the 3 splice-site junction (Arredondo-Vega et al., 1994). The presence of mRNA with normal splicing indicated that one or both mutations were somewhat leaky, and the difference in phenotype between the siblings was attributed to genetic variation in splicing efficiency. Although it would not be surprising to find that there are modifying genes, in this family the more severely affected sibling was retrospectively diagnosed as having a hepatoblastoma at the time of diagnosis of immunodeficiency. Interestingly, this tumor manifested trisomy of chromosome 20, characteristic of hepatoblastomas, and this is the chromosome on which the ADA gene resides. The presence of the tumor could well have contributed to the earlier development of immunological manifestations (Umetsu et al., 1994).

SOMATIC MOSAICISM: DE NOVO AND BY REVERSION TO NORMAL OF INHERITED MUTATIONS

Somatic mosaicism is as a modifier of phenotype, most commonly in autosomal dominant disorders. In such cases, somatic mosaicism is caused by occurrence of a de novo mutation at some point during embryogenesis, resulting in a proportion of cells that carry a mutation and a proportion that are normal. Individuals with somatic mutation in general have a milder phenotype, but, if the mutation is present in their germ cells, their offspring, to whom they transmit the mutation, carry the mutation in all cells and have a more severely affected phenotype. Somatic mosaicism is more difficult to identify for autosomal recessive disorders and has most commonly been found in relatively common disorders such as cystic fibrosis and thalassemia. We have identified somatic mosaicism in two patients with ADA deficiency (Hirschhorn et al., 1994b, 1996); in one of them it had occurred by reversion to normal of an inherited mutation. In both patients, mosaicism was uncovered during investigations directed at understanding why there had been improvement, rather than deterioration, over time of the clinical phenotype despite the absence of any form of continuing or definitive therapy. In the first patient, somatic mosaicism was demonstrated by isolation of B-cell lines that expressed ADA and lacked one of the two mutations (c.33 + 1G>C) carried by this child (but retained the p.Arg101Gln mutation), as well as by demonstration of absence of the splice-site mutation in a proportion of peripheral blood DNA. DNA

was not available from the parents, and we therefore could not prove or disprove that mosaicism had resulted from the usual mechanism of a de novo mutation during embryogenesis (Hirschhorn et al., 1994b).

In the second child we determined that somatic mosaicism had occurred because of the unexpected reversion to normal of an inherited point mutation (Hirschhorn et al., 1996). A prior sibling had died of an immunodeficiency disorder that would now be categorized as SCID but was designated Nezeloff syndrome by his physicians at the time. Each parent carried a different point mutation at the ADA locus, each of which had been identified in other ADA-deficient patients with SCID (maternal = p.Arg156His; paternal = c.478 + 1G>A). However, the child's concentrations of abnormal metabolites were relatively low and residual ADA activity in peripheral blood cells was relatively high. Moreover, instead of deteriorating without therapy, which had been refused for religious reasons, the child progressively improved clinically. We identified multiple B-cell lines that expressed ADA and determined that these carried only the paternal mutation. However, the maternal chromosome that originally bore the maternal mutation (p.Arg156His) was still present in these B-cell lines, as evidenced by the presence of a unique maternally derived RFLP linked to the mutation. Approximately 15 of the clones isolated from peripheral blood also contained the maternal marker but did not carry a mutation, confirming that reversion had occurred in vivo. This detectable reversion to normal probably involved a selective advantage for survival of revertant cells. Whether or not there was an additional specific mechanism increasing the frequency of reversion at specific sites remained to be investigated.

Reversion to normal in additional cases of ADA deficiency has now been reported (Ariga et al., 2001b). In one case, probable reversion of the paternal p.Gln119X mutation was demonstrated in a polyclonal population of herpes virus saimiri-immortalized T cells that expressed half the normal levels of ADA. Revertant cells were not recovered from the peripheral blood of the patient and therefore could have been an in vitro event. The observation of the revertant was made after the patient was started on PEG-ADA treatment, although there is evidence supporting the occurrence of the reversion event in vivo, because the dAXP levels in the patient's red cells at diagnosis were markedly lower than those expected in ADA-deficient patients carrying the same mutation. In addition, ADA activity was detectable at diagnosis at ~8 nmol/min/10^8 cells and decreased to ~3 nmol/min/10^8 cells after PEG-ADA, which could be explained by the reduction in numbers of the revertant, ADA-expressing cells that lost their selective advantage over the remaining cells after the initiation of enzyme replacement treatment. In the second case, reversion of a p.Arg235Gln maternal mutation was detected; however, because the sample studied was a monoclonal T-cell line, it could not be excluded that the reversion had occurred in vitro (Ariga et al., 2001b).

More recently, somatic mosaicism was demonstrated in a 16-year-old patient from a family in which ADA deficiency was to due to homozygosity for an intronic mutation in the last splice acceptor site of the ADA gene (c.1079-15T>A). Aberrant splicing due to the original mutation resulted in change of the last four ADA residues and added a stretch of 43 amino acids that rendered the protein unstable. The genotypic characteristics of the mosaic patient were investigated because he had greater residual immune function and less elevated erythrocyte deoxyadenosine nucleotides than his 4-year-old affected sister. Studies revealed that the patient's T cells and EBV-B cell line had 75 percent of normal ADA activity and ADA protein of normal size by Western blot. In addition, DNA from peripheral blood mononuclear cells showed two mutant ADA alleles, both carrying the c.1079-15T>A splice-site mutation, but one with an acquired deletion of the 11 adjacent nucleotides (c.1079-4_-14del), which suppressed aberrant splicing and restored protein expression and function. Interestingly, enzyme replacement therapy with PEG-ADA was also in this case followed by a reduction of ADA activity in T cells, as well as a marked decrease of the peripheral blood lymphocytes carrying the "second-site" revertant allele (Arredondo-Vega et al., 2001).

In vivo reversion of an p.Arg156His mutation was also found in purified $CD8^+$ T cells from a patient who presented at 5 years of age with clinical signs of combined immunodeficiency and chronic lung disease, a presentation similar to that of delayed-onset ADA-deficient patients. However, peripheral blood lymphocyte counts and serum immunoglobulin levels were normal in this patient. Of note, most peripheral blood lymphocytes were $CD8^+$ T cells that had normal ADA enzyme activity. Functional immune evaluation, however, showed defective lymphocyte proliferation to mitogens and antigens, as well as lack of antibody responses to pneumococcal polysaccharide vaccination. The contribution of the revertant cells to the delayed-onset phenotype of this patient is unclear; however, it can be concluded that even in the presence of high numbers of ADA-expressing lymphocytes, detoxification effects compatible with normalization of lymphocyte populations and function may not be achieved (Liu et al., 2009).

Reversion to normal of inherited mutations has now been reported in several different disorders, including dermatological and metabolic disorders in addition to various immunological disorders additional to ADA deficiency. These disorders include X-linked SCID, Wiskott-Aldrich syndrome, Fanconi syndrome, Bloom syndrome, tyrosinemia, and epidermolysis bullosa (reviewed in Hirschhorn, 2003; Wada and Candotti, 2008). Mosaicism with or without reversion should be suspected when it occurs in patients with atypical or a mild presentation of immunodeficiency. Reversion is probably a more frequent occurrence in other disorders as well as in those reported, but is most easily ascertained in disorders such as those involving hematopoietic cells and skin, where phenotypic reversion can most easily be detected.

THE HUMAN PNP GENE

The human PNP gene is encoded by a single structural gene that has been assigned to human chromosome 14q13 by somatic cell hybridization techniques (Ricciuti and Ruddle, 1973) and by gene dosage studies (Aitken and Ferguson-Smith, 1978; George and Francke, 1976). Further studies have localized the human PNP gene to 14q13.1. The gene is approximately

7.5 kb in length and contains six exons (Williams et al., 1984). A cDNA of 1.7 kb has been cloned that contains a 289-codon open reading frame, encoding a 32-kDa protein (Goddard et al., 1983). The promoter of the human PNP gene in 2 kb of the 5 flanking region contains a TATA box, an inverted CCAAT sequence, and two GC-rich regions within a 216-bp segment (Jonsson et al., 1991). Interestingly, although PNP is ubiquitously expressed in human tissues, the PNP promoter does not conform to a normal GC-rich constitutively expressed housekeeping promoter. An enhancer element was identified in the first intron of the human PNP gene (Jonsson et al., 1994).

MUTATIONAL ANALYSIS OF HUMAN PNP GENE

Diagnosis of PNP deficiency is established by demonstrating markedly reduced or absent PNP enzyme activity. Nevertheless, demonstrating the defect in the PNP gene is extremely important as it helps confirm the diagnosis, enables genetic counseling, improves understanding of the disease pathogenesis, and possibly even determines treatment options. Only one human PNP gene variant has been reported, although a pseudogene on chromosome 2 was detected by RefSeq. Sequencing of *PNP* has not been confounded by this putative pseudogene (Roifman, unpublished). Results of mutation analysis in reported patients are summarized in Figure 14.6 and Table 14.8.

The first patient to be diagnosed with PNP deficiency was also the first in whom a *PNP* gene mutation was identified (Giblett et al., 1975). The patient suffered from recurrent respiratory tract infections, diarrhea, disseminated varicella, and anemia and had no measurable PNP activity in her erythrocytes. A homozygous c.265G>A mutation was found in exon 3, putatively causing a p.Glu89Lys amino acid substitution (Williams et al., 1987). The c.265G>A mutation was also detected on one allele of another patient, together with a c.520G>C mutation that putatively caused a p.Ala174Pro substitution (patient #4, Markert et al., 1997).

A c.181G>T transversion at the terminal nucleotide of exon 2 causing aberrant splicing, with skipping of exon 2 and production of a truncated polypeptide, was found in one allele of a PNP-deficient patient (Andrews and Markert, 1992). This female patient suffered from severe varicella and postinfectious encephalopathy. PNP activity in her EBV-transformed lymphoblastoid cell line was undetectable. Sequencing of her genomic DNA demonstrated that the other allele carried a deleterious c.701G>C mutation in exon 6 that putatively caused a p.Arg234Pro substitution (Markert et al., 1997). The latter mutation was also found in three other PNP-deficient patients. One of these patients presented with recurrent infections from 22 months of age, followed by severe varicella, chronic enteroviral meningoencephalitis, and ataxia. PNP activity in her EBV-transformed lymphoblastoid cell line was only 0.8 percent of normal. In addition to the c.701G>C mutation, she also carried a c.383A>G mutation that putatively caused a p.Asp128Gly substitution (Aust et al., 1992). This patient had several polymorphic changes in her DNA, including double A in the 3′ untranslated region (903), two tandem copies of a 10 base pair sequence in the 5′ flanking region (c.-41_-50dup), as well as c.60T>C and c.171T>C nucleotide substitutions that were not predicted to cause amino acid changes. Another mutation was also detected in the patient, a c.151A>G nucleotide substitution in exon 2, putatively causing a p.Ser51Gly change. However, the c.151A>G mutation expressed in COS cells did not affect PNP activity in these cells (Aust et al., 1992).

Another patient with the c.701C>G mutation also carried a splice mutation caused by a G-to-A substitution 18 base pairs upstream of exon 4 that created an alternative splice site and unstable mRNA. Despite this, small amounts of normal transcripts were produced from the normal splice site (Markert et al., 1997). This patient was diagnosed with cerebral palsy at 2 years of age but suffered from significant infections only after 4 years of age. Moreover, PNP activity in her erythrocytes was approximately 3 percent of normal (Broome et al., 1996). Thus, it was hypothesized that residual PNP activity contributed to the relatively mild phenotype (Markert et al., 1997). The c.701G>C mutation was also found in association with c.385_387delATC in exon 4 (patient #6, Markert et al., 1997).

The c.569G>T mutation, putatively causing a p.Gly190Val amino acid substitution, was found in one PNP-deficient patient (patient #5, Markert et al., 1997). This mutation was suspected to be homozygous; however, the authors could not exclude the possibility that the second allele carried a large deletion.

A frameshift mutation of the PNP gene was described in a child who presented at 6 months of age with neurological abnormalities that were followed by several episodes of pneumonia and sepsis. Analysis of the patient's DNA revealed that the frameshift was caused by a c.730delA deletion in exon 6 on one allele, while the other allele carried a c.575A>G transition in exon 5, putatively causing a p.Tyr192Cys substitution (Pannicke et al., 1996).

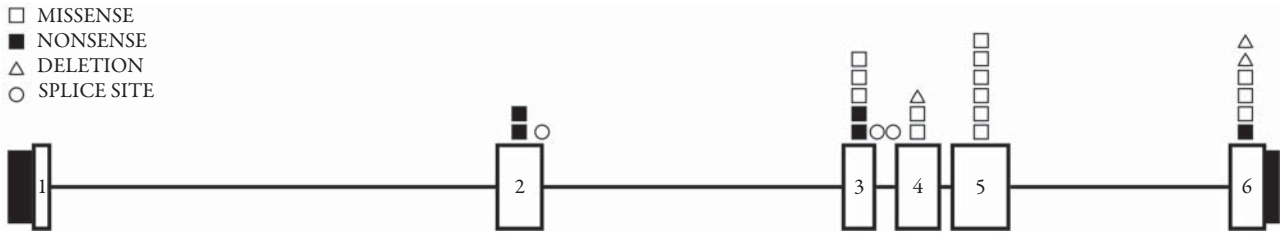

Figure 14.6 Mutations at the PNP locus.

Table 14.8 MUTATED ALLELES OF THE PURINE NUCLEOSIDE PHOSPHORYLASE (PNP) GENE

EFFECT	SITE	SEQUENCE VARIATION	REFERENCE
A. Missense Mutations			
p.Gly71Glu	exon 3	c.212G>A	Parvaneh et al., 2008
p.Leu73Pro	exon 3	c.218T>C	Baguette et al., 2002
p.His86Arg	exon 3	c.257A>G	Walker et al., 2011
p.Glu89Lys	exon 3	c.265G>A	Williams et al., 1987
p.Ala117Thr	exon 4	c.349G>A	Tabarki et al., 2003
p.Asp128Gly	exon 4	c.383A>G	Markert et al., 1997
p.Pro146Leu	exon 4	c.437C>T	Alangari et al., 2009
p.Gly156Ala	exon 5	c.467G>C	Moallem et al., 2002
p.Phe159Val	exon 5	c.475T>G	Parvaneh et al., 2008
p.Ser163Pro	exon 5	c.487T>C	Al-Saud et al., 2009
p.Tyr166Cys	exon 5	c.497A>G	Tsuda et al., 2002
p.Ala174Pro	exon 5	c.520G>C	Markert et al., 1997
p.Gly190Val	exon 5	c.569G>T	Markert et al., 1997
p.Tyr192Cys	exon 5	c.575A>G	Pannicke et al., 1996
p.Arg234Pro	exon 6	c.701G>C	Aust et al., 1992
p.His257Arg	exon 6	c.770A>G	Walker et al., 2011
B. Nonsense Mutations			
p.Arg24X	exon 2	c.70C>T	Sasaki et al., 1998
p.Arg57X	exon 2	c.172C>T	Dalal et al., 2001
p.Arg67X	exon 3	c.199C>T	Walker et al., 2011
p.Gln82X	exon 3	c.244C>T	Somech et al., 2012
C. Splice-Site Mutations			
deletes exon 3 & frameshift	exon 2	c.181G>T	Andrews & Markert, 1992
deletes exon 3 & frameshift	IVS 3	c.285+1G>A	Dalal et al., 2001
+16 bp & frameshift	IVS 3	c.286-18G>A	Markert et al., 1997
D. Deletions/Insertions			
p.Ile129del	exon 4	c.385_387delATC	Markert et al., 1997
p.Gly156fsX170	exon 6	c.468delA	Baguette et al., 2002
p.Asn243fsX261	exon 6	c.730delA	Pannicke et al., 1996
E. Polymorphisms (SNPs) at the PNP Locus			
Dup 10bp	5′ UTR	c.-41_-50dup CGGATCGGAG	Williams et al., 1984, 1987
Synonimous	exon 2	c.60T>C	Aust et al., 1992
p.Ser51Gly	exon 2	c.151A>G	Aust et al., 1992
Synonimous	exon 2	c.171T>C	Aust et al., 1992
p.Val217Ile	exon 5	c.649G>A	Moallem et al., 2002
	3' UTR	c.*34insA	Williams et al., 1984, 1987

NCBI Reference Sequence: NM_000270.3

A homozygous c.70C>T mutation in exon 2 that putatively caused an p.Arg24Stop substitution was found in a Japanese male patient who suffered from recurrent urinary tract infections after birth and abnormal T-cell function. His erythrocytes had 2.3 percent PNP activity, with no immunoreactive PNP (Sasaki et al., 1998). We also identified the p.Arg24Stop mutation in a patient born to a Japanese mother and Caucasian father. This patient suffered from mild gross motor developmental delay and ataxia. At 2 years of age he developed erythrocyte aplasia after prolonged parvovirus infection, followed by recurrent herpes zoster and herpes simplex infections. PNP activity in his erythrocytes was not detectable. The patient inherited the c.172C>T transition from his mother, while the paternal allele carried a c.285 + 1G>A transition in intron 3, which resulted in skipping of exon 3, and addition of 29

nonrelevant amino acids before a premature stop codon (Dalal et al., 2001).

A homozygous c. 467G>C mutation in exon 5 that putatively caused a p.Gly156Ala substitution was reported in a Bahamian girl. She suffered from respiratory infections, mucocutaneous candidiasis, failure to thrive, hypotonia, and severe developmental delay from 8 months of age. Her T-cell numbers were markedly reduced, but cells did respond to stimulation. PNP activity in her erythrocytes was essentially absent. An additional homozygous c.649G>A mutation was found that putatively caused a p.Val217Ile substitution, but with no effect on PNP function (Moallem et al., 2002).

A homozygous c.349G>A mutation that putatively caused a p.Ala117Thr substitution was reported in a Tunisian girl who suffered from progressive spastic paraplegia from the first year of life. She also developed recurrent respiratory infections at 3 years of age and died of malignant lymphoma. Her younger sister presented at 6 months of age and suffered a similar course. PNP activity in erythrocytes and white blood cells was absent (Tabarki et al., 2003). The same mutation was reported in Turkish girl who suffered from motor and mental developmental delay in the first 2 years of life, recurrent respiratory tract infections, and failure to thrive. She died at 2.5 years of age after severe bronchopneumonia and herpes virus infection. PNP activity in her skin fibroblasts was less than 0.3 percent of normal (Ozkinay et al., 2007).

A homozygous c.212G>A substitution resulting in a putative p.Gly71Glu substitution was identified in a 6-year-old Iranian boy who suffered from delayed motor development and repeated respiratory infections from 4 years of age. He died at 6 years of age from pulmonary infection (Parvaneh et al., 2008). PNP activity in his cells was less than 2.5 percent. Another Iranian boy was found to have a homozygous c.475T>G mutation that putatively caused a p.Phe159Val substitution. This patient was well until 7 years of age, when he developed sinopulmonary infections, autoimmune arthritis, Coombs-positive hemolytic anemia, and spastic paraparesis. He died a year later of progressive multifocal leukoencephalopathy due to JC virus infection (Parvaneh et al., 2007). Although the PNP activity in his cells was not determined, Phe159 forms an essential part of the active catalytic site of the enzyme, and the mutation of this residue is therefore expected to significantly reduce the enzyme activity (Parvaneh et al., 2008).

Recently PNP deficiency was reported in two presumably unrelated girls from Saudi Arabia who suffered from severe lymphopenia and neurological abnormalities at an early age (Alangari et al., 2009). Sequencing of the PNP gene in one demonstrated a c.437C>T change putatively resulting in a p.Pro146Leu amino acid substitution. Expression of the mutated enzyme in *E. coli* indicated that the mutation greatly reduced, but did not completely eliminate, PNP activity.

Additional missense (p.His86Arg, p.Ser163Pro, p.His257Arg) and nonsense mutations (p.Arg67X, p.Gln82X) have been recently reported (Al-Saud et al., 2009; Somech et al, 2012; Walker et al., 2011).

Polymorphic changes predicted to be silent or to cause substitution of amino acids in the PNP gene without affecting PNP enzymatic activity are listed in Table 14.8. Additional polymorphisms in the PNP gene were recently described in European and Indigenous Americans (Yu et al., 2003). The contributions of these polymorphisms to various metabolic processes and neurological abnormalities are being investigated (De Chaudhuri et al., 2008; Tumini et al., 2007).

The mutations in PNP patients described above suggest that residual PNP enzyme activity may lead to less severe manifestations; however, establishing clear genotype–phenotype correlation and prediction of prognosis in this disease will require studying significantly larger numbers of patients. In contrast to ADA deficiency, no PNP patients with somatic mosaicism have been described, possibly because of the smaller number of PNP patients studied or due to a reduction in the selective advantage that PNP-proficient cells possess in a PNP-deficient environment.

STRATEGIES FOR DIAGNOSIS

Based upon the emerging phenotype of late- and adult-onset immunodeficiency disease due to ADA deficiency, any individual with lymphopenia of unexplained etiology and frequent infections of any type, with or without autoimmunity, should be tested for ADA deficiency; in this setting an initial screening assay of erythrocytes should be followed by an assay of ADA in nonerythroid cells.

Diagnosis of ADA deficiency in immunodeficient patients can be made by enzyme assay of several easily available cell types, including erythrocytes, lymphocytes, EBV-transformed B cells, and fibroblasts. If the patient has received a transfusion, erythrocytes are unreliable, and assay of a transformed B-cell line or of fibroblasts should be used. If the diagnosis is made by assay of red blood cells, an assay of lymphocytes and/or fibroblasts should also be performed to rule out the (unlikely) possibility of partial ADA deficiency, which would not be expected to give rise to immunodeficiency. Alternatively, analysis of DNA and identification of previously reported deleterious mutations can be performed. While there may not be sufficient lymphocytes for assay in the initial blood sample, there is usually sufficient DNA in the buffy coat that is removed prior to assay of red cells, and that should be saved and can be used to sequence all exons and flanking regions. Determination of deoxyATP and of deoxyadenosine in urine can also aid in diagnosis.

A variety of assays are available to measure the hydrolytic deamination of adenosine (Zielke and Sueltre, 1971). We have not found assays measuring release of ammonia to be reliable. For initial screening, we routinely utilize a linked spectrophotometric assay in which uric acid is the final end product (Edwards et al., 1971; Hirschhorn, 1979b). This assay is relatively sensitive, rapid, and easily performed. A second spectrophotometric method measuring the conversion of adenosine to inosine by loss of absorbance at the peak for adenosine is also simple and widely used, but it is much less sensitive because of the limitation in the concentration of substrate and in the amount of cell lysate that can be included in the assay. The most sensitive and specific assays are those

that utilize radiolabeled adenosine as substrate in the presence and absence of the inhibitor EHNA and separate the products from the substrate by methods such as thin-layer chromatography (Hirschhorn, 1979b). The amount of enzyme activity that is inhibited by EHNA measures the activity resulting from the ADA locus affected in ADA-deficient immunodeficiency. The alternate isozyme of ADA is encoded by a distinct locus not affected in ADA-deficient immunodeficiency. Although this locus accounts for only 1 to 2 percent of ADA activity in normal cells, its presence can confuse detailed characterization of residual ADA in the diagnosis of ADA deficiency. We have also utilized an HPLC assay using unlabeled adenosine and standard methods of separation (Hirschhorn et al., 1982), but this requires expensive machinery not commonly available. However, this assay could be easily automated with current available technology; it does not require radioactivity, and it is sensitive. Other methods amenable to automation have also been described (Carlucci et al., 2003). Among these, tandem mass spectrometry can be applied to newborn screening and has been demonstrated to be able to identify newborns with early- and delayed-onset ADA-SCID through analysis of dried blood spots collected at birth (Azzari et al., 2011; la Marca et al., 2012). Interestingly, SCID newborn screening methods based of TRECs may miss delayed/late-onset ADA-deficient cases (la Marca et al., 2012; Speckman et al., 2012), which supports the application of alternative screening methods, such as tandem mass spectrometry and KRECs.

PNP deficiency should be suspected in patients presenting with recurrent bacterial, viral, or fungal infections, associated with reduced T-cell numbers and function. Unexplained neurological abnormalities associated with reduced T-cell immunity should also raise the possibility of PNP deficiency, although other etiologies including DiGeorge syndrome, ADA deficiency, XLF-Cernunnos and DNA ligase 4 deficiency, dyskeratosis congenita, Bloom syndrome, ataxia-telangiectasia, Nijmegen breakage syndrome, and others should be considered. A decreased uric acid concentration in the blood (<2 mg/dL, but usually <1 mg/dL) and/or urine is an important clue to the diagnosis of PNP deficiency; however, severe neurological manifestations associated with hypouricemia can also be caused by molybdenum cofactor deficiency and phosphoribosyl pyrophosphate synthetase deficiency. Liver disease, malnutrition, proximal renal tubular disease, xanthinurea, as well as other conditions and medications can reduce the concentration of uric acid in the blood and urine. Increased concentrations of dGTP in erythrocytes and high levels of inosine, deoxyinosine, guanosine, and deoxyguanosine in the serum and urine are characteristic for PNP deficiency (Cohen et al., 1978; Hershfield and Mitchell, 2001), but these assays are not readily available in most laboratories.

The diagnosis of PNP deficiency is usually confirmed by demonstrating markedly reduced (<5 percent) or absent PNP enzyme activity in erythrocyte lysates (or other cells if the patient recently received red blood cell transfusions) by radiochemical or spectrophotometric assays. Measurements of PNP activity can be performed in dried blood spots using reversed-phase HPLC (van Kuilemburg et al., 2010). ADA activity is often measured in the same sample used for PNP detection, because of the significant clinical and immunological overlap between these two immunodeficiency diseases and as an internal control to confirm that handling of the sample did not affect enzyme activity. Measuring activity of both enzymes is particularly important with the linked spectrophotometric assay because the ADA assay depends upon endogenous PNP activity, and therefore an absence of PNP can initially be misinterpreted as ADA deficiency.

PRENATAL DIAGNOSIS AND CARRIER DETECTION

Prenatal diagnosis of ADA deficiency has been made by enzyme assay on chorionic villous samples, amniotic cells, and fetal blood (Durandy et al., 1982; Hirschhorn, 1979b, 1979c; Hirschhorn et al., 1975, 1992; Linch et al., 1984; Simmonds et al., 1983, and Hirschhorn, unpublished). We have performed prenatal diagnosis in over 20 cases by enzyme assay of amniotic cells and correctly diagnosed the expected 25 percent to be affected. Predictions were confirmed either by postnatal ADA assay of erythrocytes and/or identification of mutations on both chromosomes in amniotic cells; infants predicted to be unaffected had ADA activity by assay of erythrocytes after birth. There was a clear separation between affected fetuses and fetuses at risk but not affected. However, homozygous normals versus carriers could not be definitively identified by enzyme assay in cases where we could identify carriers by heterozygosity for a mutation.

DNA-based diagnosis can definitively diagnose carriers, affected subjects, and normals in a setting where both ADA mutant alleles are known; this is feasible if the mutations have been studied in a previously affected child in the family or can be rapidly determined in the parents or samples preserved from a deceased affected child. Although RFLPs, when informative, could in theory also be used for diagnosis, we suggest that the AluVpA polymorphism described upstream of the first exon not be used for diagnosis, as we have identified a crossover in this region (Hirschhorn et al., 1994b). Direct detection of the specific pathological mutations or enzyme assay is preferable, and identification of the mutations present can be performed easily, given the availability of PCR and rapid sequence analysis.

Prenatal diagnosis can be valuable not only if parents wish to terminate an affected pregnancy but also for facilitating therapy of an affected infant after birth. A number of families have chosen to carry the pregnancy of an affected child to term (Jiang et al., 1997). HLA typing has then allowed for rapid bone marrow transplantation (both histocompatible and T-depleted haploidentical) before the onset of infections (Pollack et al., 1983). Prenatal diagnosis has also allowed for harvesting of cord blood for gene therapy (Kohn et al., 1995, 1998).

Similarly to ADA deficiency, both amniotic cell culture and chorionic villus sampling have been used successfully to identify affected fetuses by assessing PNP enzymatic activity (Carapella De Luca et al., 1986; Kleijer et al., 1989; Perignon et al., 1987). Direct PNP gene sequencing was also shown to

Table 14.9 **THERAPEUTIC OPTIONS FOR ADA DEFICIENCY**

1. Preventive
a. Prenatal diagnosis with selective pregnancy termination
b. Prenatal diagnosis to assist postnatal therapy—HLA typing, germ-free delivery, immediate antibiotics, etc.
c. Preimplantation diagnosis with selective implantation of nonmutant, histocompatible embryos to serve as hematopoietic stem cell donors at birth
2. Hematopoietic Stem Cell Transplantation
a. Potential donors
i. Histocompatible-related donor (usually sibling)[a]
ii. Haploidentical-related donor (usually parent)
iii. Matched unrelated donor
b. Potential sources of hematopoietic stem cells
i. Bone marrow
ii. Mobilized peripheral blood
iii. Cord blood
3. Enzyme replacement
a. Partial exchange transfusion (no longer used)
b. PEG-conjugated calf intestinal ADA (Adagen)
4. Gene therapy; introduction of retrovirally carried correct gene sequence into:
a. Cultured expanded T cells
b. $CD34^+$ stem cells
i. Cord blood
ii. Bone marrow

[a]Therapy of choice, if available

accurately identify the PNP status of a fetus from a family with a previously identified PNP-deficient sibling (Roifman, unpublished).

Determination of heterozygous carriers can be attempted by assay of ADA in erythrocytes and demonstration of anomalous inheritance within the family of the normal ADA polymorphism (Chen et al., 1974; Hirschhorn, 1992; Hirschhorn et al., 1975; Scott et al., 1974). However, there is a large range of normal ADA activity; values follow a normal distribution only after log conversion. Most obligate carriers demonstrate, as expected, approximately half-normal ADA activity, but approximately 10 to 20 percent of carriers fall within the normal range, and 1 percent of normals have activity in the carrier range. Most troubling are those individuals at risk for being carriers who are at the borderline of normal. DNA-based mutation detection now offers a definitive test in most families. In the case of a pregnancy at risk for SCID of unknown etiology, a prenatal diagnosis of SCID can be made by examining lymphocytes in fetal blood, at which time ADA deficiency can be diagnosed with the erythrocytes obtained. Preferably, ADA can be assayed in cultured amniocytes. Clearly, a consensus is needed as to the most efficient, sensitive, and specific strategies to be used for prenatal diagnosis of SCID that would avoid fetal blood sampling.

Carriers for PNP deficiency frequently have decreased PNP activity in red cell and white cell lysates. If the mutations of the affected offspring are known, DNA-based mutation analysis will determine carrier status in the family.

TREATMENT AND PROGNOSIS

Several clinical management options are available for ADA deficiency. These are listed in Table 14.9 and include both prevention (e.g., prenatal diagnosis with selective pregnancy termination) and therapeutic approaches based on hematopoietic stem cell transplantation (HSCT), enzyme replacement, and gene therapy (Gaspar et al., 2009). In contrast to ADA deficiency, management of PNP deficiency is often limited to preventive and supportive measures. HSCT transplants are not commonly performed because of low success rates, while enzyme or gene therapies are not yet available.

Bone Marrow/ Hematopoietic Stem Cell Transplantation

Allogeneic HSCT from a histocompatible sibling or other close relative in inbred communities is an established form of therapy for ADA deficiency (Amrolia et al., 2000; Antoine et al., 2003; Bertrand et al., 1999; Boulieu et al., 1988; Buckley et al., 1986, 1993, 1999; Cancrini et al., 2010; Chen et al., 1978; Fischer, 1992; Fischer et al., 1986, 1990; Friedrich et al., 1985;

Gaines et al., 1991; Haddad et al., 1998; Hassan et al., 2012; Markert et al., 1987; Parkman, 1991; Parkman et al., 1975; Rubocki et al., 2001; Serana et al., 2010; Silber et al., 1987; Wijnaendts et al., 1989; Zegers and Stoop, 1983) (Honig et al., 2007). This therapeutic maneuver has the greatest efficacy, does not necessarily require prior marrow ablation or permanent ongoing therapy, and has a relatively low morbidity and mortality. After successful HSCT, deoxyadenosine-based metabolites are dramatically lower than in untreated patients but are still detectably higher than normal (Hirschhorn et al., 1981). The diminution of adenosine concentrations is not as dramatic, and this observation is to be expected based on the fact that the ADA enzyme has a greater affinity (lower Km) for deoxyadenosine than for adenosine. Surprisingly, adenosine can persist in plasma at very high levels and this remains a concern based on the observation that in ADA-deficient mice, elevated adenosine has significant effects (see the section "Animal Models" below), . Additionally, SAH hydrolase activity remains low, reflecting retention of increased amounts of metabolites (Hirschhorn et al., 1981). The incomplete metabolic correction may explain the persistence of nonimmunological manifestations in transplanted patients such as sensorineural deafness and cognitive and behavioral abnormalities (Albuquerque and Gaspar, 2004; Rogers et al., 2001; Tanaka et al., 1996), despite the fact that the bony abnormalities disappear rapidly. Genetic deficiency of SAH hydrolase is now known to result in mental deterioration, hypotonicity, and myopathy as well as multiple biochemical aberrations, including hypermethionemia, but without signs of immunodeficiency (Baric et al., 2004). The ability of dietary manipulation to reverse the metabolic abnormalities and apparently the mental deterioration in SAH-deficient patients (Baric et al., 2004) raises the need to assess the correction of SAH pathways in post-BMT ADA-deficient patients and offers a possible role for dietary manipulation in ADA-, but also PNP-, deficient patients with cognitive defects. Finally, it is also not known whether transplantation from an ADA-heterozygous carrier has different effects than from a homozygous normal. Metabolites have not been reported in patients transplanted following marrow ablation sufficient to result in replacement of all hematopoietic elements with donor-derived precursors.

Unfortunately, in an outbred population with small family size, as typically exists in the United States, fewer than 25 percent of patients have an HLA-matched related donor. Haploidentical transplantation from a related donor has higher morbidity and mortality in all forms of SCID than HLA-matched transplantation. It usually requires pretreatment myeloablation, but it is performed in the absence of preparative chemotherapy in some centers (Buckley et al., 1999) and can result in permanent engraftment (see Chapter 60). The European Bone Marrow Transplantation working party (EMBT) and the European Society for Immunodeficiency have published the analysis of a multicenter retrospective study on the outcome of BMT in 106 patients with ADA-SCID. BMT from matched sibling and family donors had significantly better survival (86% and 81%) in comparison with BMT from matched unrelated (66%; $P < .05$) and haploidentical donors (43%; $P < .001$). The vast majority of surviving patients achieved recovery of both cellular and humoral immunity (Hassan et al, 2012). Currently, therefore, it appears that ADA-deficient patients are poorer candidates for haploidentical transplantation than other forms of SCID, in which the average survival is greater than 54 percent (Antoine et al., 2003). The EBMT/ESID study showed that patients who received unconditioned transplants had superior overall survival in comparison with myeloablative procedures (81% vs 54%; $P < .003$). Reduced intensity conditioning protocols are being tested to reduce toxicity while achieving adequate donor engraftment (Cancrini et al, 2010). While BMT continues to be an intensive procedure with potential severe adverse effects, studies have shown that it provides enhanced immune reconstitution in comaparison with ADA enzyme replacement therapy (Serana et al., 2010; Kanegane et al., 2013).

Enzyme Replacement Therapy

Enzyme replacement therapy for ADA-deficient SCID does not require uptake of the enzyme into cells. Therapy is based, first, upon the fact that metabolites are present in plasma and body fluids, where they can be degraded by administered enzyme present in the plasma; second, additional toxic metabolites such as dATP are in equilibrium with the precursors in plasma (Hershfield, 1995a, 1995b, 2000; Hershfield et al., 1987, 1993; Hirschhorn et al., 1980a; Hopp et al., 1985; Ochs et al., 1992; Polmar et al., 1976; Weinberg et al., 1993).

Therefore, lowering of substrate concentrations in plasma results in lowering of intracellular concentrations of metabolites as well (Fig. 14.7). The first attempts at enzyme therapy utilized partial exchange transfusion with normal, irradiated erythrocytes, based on the fact that erythrocytes have transport sites for both adenosine and deoxyadenosine as well as containing adenosine deaminase. Partial exchange transfusion lowered metabolite concentrations dramatically, restored normal growth and development, and prolonged survival in at least six patients. However, this treatment did not alter immunological responsiveness in most patients; moreover, any improvement in immunological parameters was often short-lived (Ochs et al., 1992). Additionally, transfusion carried the risk of transmission of infectious agents and resulted in iron overload. Three patients first treated with transfusion therapy survived until over 10 years of age and were then placed on enzyme replacement therapy with intramuscular bovine ADA (see below); two of these are siblings were still alive at approximately 20 years of age. Of note, infusions of autologous red blood cells loaded with bovine ADA have been used in one patient with reported beneficial results (Bax et al., 2007).

The development of PEG-ADA enzyme replacement therapy (polyethylene-glycol-modified calf intestinal ADA; pegadamase bovine [Adagen]) has completely replaced the original enzyme replacement treatments. The modification, involving the addition of PEG to lysine residues, is designed to mask the enzyme so that its half-life is markedly prolonged. While dosages recommended are from 15 to 30 U/kg of body weight weekly, regimens may be individualized, based on monitoring

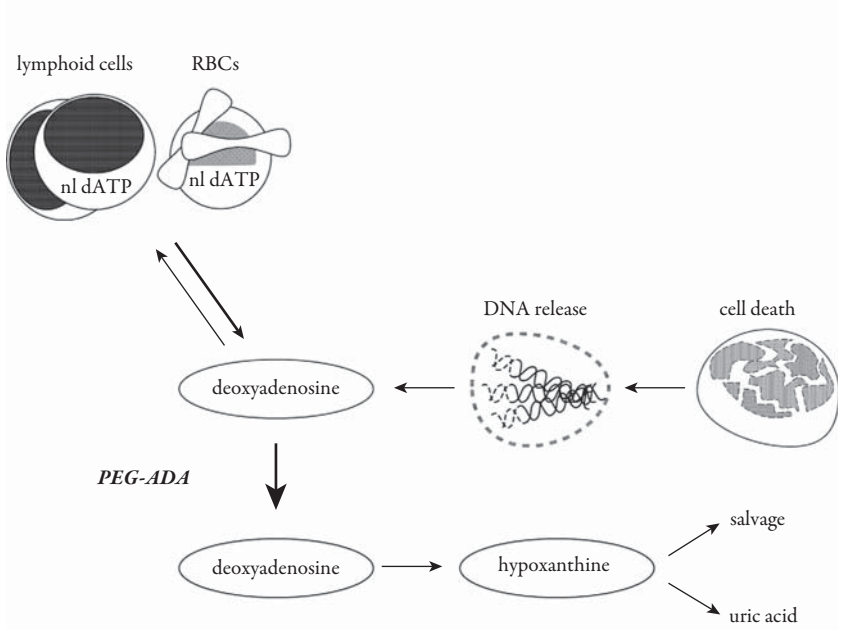

Figure 14.7 ADA deficiency: effects of enzyme therapy.

of plasma ADA activity. In practice, early-onset SCID patients are being treated with twice-weekly intramuscular injections of 30 to 60 U/kg, based on ideal body weight, for the first several months of therapy. There may be a diminished half-life of the enzyme in these very ill infants and/or poorer responses to usual doses. Monitoring of dATP, total deoxyadenosine nucleotides, and SAH hydrolase activity is recommended since the increase in deoxynucleotides and decrease in SAH hydrolase activity have been associated with a reduction of lymphocyte counts and function (Chun et al., 1993). Periodic testing for development of anti-ADA antibodies is also advisable.

PEG-ADA therapy has completely replaced partial exchange transfusion because it is safer; it can deliver greater amounts of enzyme and therefore more completely lower metabolites; and, in addition to restoring normal growth and development and prolongation of life, it results in the development of protective, although not normal, T-cell immunity in ~80 percent of the treated patients. To date, more than 180 patients have been treated with PEG-ADA worldwide, with a survival rate of approximately 80 percent (Gaspar et al., 2009). Virtually all patients on PEG-ADA develop antibodies against the bovine ADA protein that in ~10 percent of the cases are neutralizing antibodies for the function of the injected enzyme and require increasing the dosage or discontinuation in an attempt to achieve desensitization (Chaffee et al., 1992; Chun et al., 1993; Hershfield, 2004). Other complications seen in patients on PEG-ADA treatment include autoimmune hemolytic anemia and EBV-positive lymphoproliferative syndrome, which have required therapeutic attempts with HSCT. One of the patients responsive to transfusion treatment died of uncontrollable autoimmune hemolytic anemia following a viral infection (Ratech et al., 1985). A second died at 6 years of age of overwhelming varicella when exposure was not recognized in time to administer appropriate preventive therapy. Of note, elective HSCT after PEG-ADA treatment has resulted in survival of ~50 percent of cases, suggesting that the use of PEG-ADA to stabilize the metabolic status of patients after diagnosis and before transplantation can be beneficial (Gaspar et al., 2009).

Overall, although reconstitution of immunity appears to be suboptimal, the clinical response of children on PEG-ADA has been good, as measured by normal growth and development, ability to attend school, absence of opportunistic infections, and normal recovery from varicella and other viral diseases. Early mortality appears to be limited to early-onset cases with severe infectious complications. Immune reconstitution, as measured by lymphocyte counts and in vitro function, has been less complete, and ~20 percent of patients, predominantly with infantile onset of disease, show slower and lesser improvement. An increase in B cells usually precedes the increase in T cells and the appearance of mitogen responses. Although mitogen responses improve from the initial 0 to 10 percent of normal, they then fluctuate between 25 and 90 percent of normal. After the first year, most patients remain lymphopenic. Specific antibody responses have been detected in half of the patients, and half no longer receive regular intravenous immunoglobulin replacement (Hershfield, 2004). Elegant studies of two patients with ADA-deficient SCID of early onset during their first year of enzyme therapy have provided evidence that the therapy results in apparent rescue of immature lymphocyte progenitors. It was shown that mature immunocompetent lymphocytes were recruited from progenitors, recapitulating ontogeny, rather than merely expanding a small pool of relatively mature cells (Weinberg et al., 1993). Thus PEG-ADA therapy, adjusted as needed to normalize SAH hydrolase activity, resulted in the sequential appearance of $CD3^{dim}$ cells, followed by transient appearance of cells expressing both CD4 and CD8, and finally the mature

phenotype of $CD3^{bright}$ cells expressing either CD4 or CD8. These changes were observed between 3 and 5 months following the start of therapy. The appearance of mature T cells was then followed by IL-2-dependent mitogen responses and then rapidly by IL-2-independent responses and antigen-specific responses, also initially transiently IL-2 dependent. Other studies have demonstrated normalization of the antibody response, as indicated by the appearance of responses to the bacteriophage ΦX174 (Ochs et al., 1992).

Enzyme replacement therapy results in dramatic lowering of metabolites, most notably in returning the SAH hydrolase activity to normal or almost normal. The activity of SAH hydrolase appears to be the most sensitive indicator of total body concentration of toxic metabolites. The major questions remaining as to PEG-ADA therapy are whether immune function will remain improved over the years and whether the efficacy will be limited by the underlying phenotype or by a prior history of infections that have presumably destroyed a significant portion of the pool of "rescueable" lymphocyte precursors. A retrospective study designed to assess the long-term effectiveness of PEG-ADA treatment on nine ADA-deficient SCID patients (age 5 to 15) showed that despite initial improvements, the lymphocyte counts of all of the PEG-ADA–treated patients were below the normal range at all times. The study also showed that a gradual decline of mitogenic proliferative responses was evident after a few years of treatment and normal antigenic response occurred less than expected (Chan et al., 2005). In addition to the possible transitory efficacy of the treatment, the other major disadvantages of PEG-ADA therapy are its high cost, the necessity for weekly, life-long therapy (possibly requiring ongoing monitoring of metabolites for adjustment of dose), and the possibility of developing clinically significant anti-enzyme antibody. The cost and need for ongoing therapy are important although not insurmountable. A recombinant human form of the enzyme is under study and could solve or reduce the problems of anti-ADA antibody.

Gene Therapy

ADA-deficient SCID has been the pioneer disorder for the development of human gene therapy trials (see Chapter 61) (Aiuti et al., 2002; Blaese et al., 1995; Bordignon et al., 1995; Hoogerbrugge et al., 1996; Kohn et al., 1995; Onodera et al., 1998). ADA deficiency appeared to be an excellent candidate for gene therapy, based on the knowledge that the disease could be corrected by bone marrow transplantation; that manipulation of bone marrow cells ex vivo prior to transplantation is routine and could be easily adapted to allow for introduction of the ADA cDNA into cells; and that regulated expression did not appear to be required. Experiments in mice indicating that lymphoid cells containing the ADA gene have a marked advantage over ADA-deficient cells in an ADA-sufficient environment, a surrogate model for enzyme therapy (Ferrari et al., 1991), and results of bone marrow transplantation consistent with the hypothesis that endogenous ADA is more effective than exogenous ADA provided a rationale for attempts at gene therapy for ADA deficiency despite the availability of alternative modes of therapy (e.g., bone marrow transplants, PEG-ADA). The development of gene therapy was supported by its important theoretical advantages, such as avoiding the threat of graft-versus-host disease and availability to all patients. To be successful, efficient and stable gene transfer into patients' cells is a prerequisite and, ideally, genetic correction of hematopoietic stem cells should be achieved.

Gene therapy for the treatment of ADA deficiency by introduction into hematopoietic cells of a normal ADA cDNA contained in a retroviral vector has been pursued in five different countries: the United States, the Netherlands, Italy, the UK, and Japan. As of 2013, ~60 patients have enrolled in these trials, although published data are not available for all of the cases. Most patients have been treated using a retroviral vector containing the normal ADA cDNA and with ex vivo gene transfer. Very recently, trials using lentiviral vectors have begun and enrolled a few patients. In the U.S., gene therapy was initially performed in two patients using T cells expanded in vitro as the target cells (Blaese et al., 1995; Mullen et al., 1996; Muul et al., 2003) and subsequently $CD34^+$ cells isolated from the patients' cord blood in three newborns diagnosed prenatally (Kohn et al., 1995, 1998). Patients treated with T-cell gene therapy showed improvement of a series of immunological parameters (Blaese et al., 1995), although the concomitant PEG-ADA treatment makes it difficult to attribute these results to gene therapy alone. Nevertheless, this trial has demonstrated that retroviral-mediated ADA gene transfer into T cells can achieve long-term (15+ years) presence and expression of the exogenous gene (Muul et al., 2003).

The design of the first Italian trial included both peripheral blood mononuclear cells and T-cell–depleted bone marrow cells corrected with distinguishable ADA gene transfer vectors. The results from the initial two treated patients indicated that short-term marking was achieved from cells derived from peripheral blood, whereas ADA-corrected cells detected in patients after 1 year were from bone-marrow–derived cells (Bordignon et al., 1995).

While restoration of immune function through these initial experiments was incomplete or absent, the clinical and technical experience generated by these investigations was remarkable and represented a stepping stone on which more recent successes were built. Indeed, ongoing trials implementing more efficient culture condition and gene transfer vectors have recently demonstrated that gene therapy can be successful in ADA deficiency (Kohn and Candotti, 2009). Indeed, multilineage engraftment of gene-corrected cells, metabolic correction, and improvement of immune function was achieved in eight of ten ADA-deficient patients who were not treated with PEG-ADA and received genetically modified $CD34^+$ cells after preparative nonmyeloablative chemotherapy (Aiuti et al., 2002, 2009a). These results are superior to those of other groups that have treated ADA-deficient patients in the UK, United States, and Japan (Gaspar et al., 2006, 2012; Candotti et al., 2012) and suggest that additional improvements are necessary for gene therapy to become a widespread alternative treatment option for ADA deficiency. Among these, is the development of lentiviral vectors (Carbonaro et al., 2006; Mortellaro et al., 2006) that are expected to be the main gene

transfer tool in future trials. These vectors are are known to be less prone to integration near gene transcriptional start sites and are therefore considered less likely to cause insertional oncogenesis adverse events such as those occurred in gene therapy trials for X-linked severe combined immunodeficiency, chronic granulomatous disease and Wiskott-Aldrich syndrome (Aiuti et al., 2012; Cavazzana-Calvo et al., 2012).

Management of PNP-Deficient Patients

Management of PNP-deficient patients includes extensive and frequent immunological, hematological, and neurological assessments, as the clinical and laboratory features of this disease fluctuate and deteriorate over time. Intravenous immunoglobulin replacement is indicated for PNP-deficient patients, particularly when abnormal production of antibodies is demonstrated. Patients should receive prophylaxis for *Pneumocystis jiroveci* pneumonia. PNP-deficient patients who are able to produce antibodies and are not receiving intravenous immunoglobulins should be vaccinated, but not with live viral vaccines. All blood products given to PNP-deficient patients should be CMV-negative (if the patient has not had CMV infection previously) and irradiated to prevent graft-versus-host disease. Despite all these supportive measures, PNP-deficient patients invariably die in their first or second decade of life from disseminated viral infections, uncontrolled autoimmune hematological cytopenia, or malignancy. The extremely poor outcome for PNP-deficient patients has prompted attempts to correct the metabolic toxicity, thereby restoring immune function, or directly correcting the immune abnormalities.

To alleviate some of the effects of abnormal purine metabolism, deoxycytidine, which bypasses ribonucleotide reductase inhibition, was given together with tetrahydrouridine to inhibit deoxycytidine deaminase (Markert, 1991). This combination did decrease dGTP concentrations and increase GTP; however, the two treated PNP-deficient patients died of infections. One patient received uridine to correct potential pyrimidine starvation with no significant immune benefit. Another patient, who received guanine to increase intracellular GTP, experienced only a brief improvement in T-cell function. Similarly, treatments with adenine, uridine, hypoxanthine, or allopurinol did not improve lymphocyte responses to stimulation (Staal et al., 1980a).

PNP enzyme replacement with repeated erythrocyte transfusions, similar to the treatment initially used for ADA deficiency, was given in an attempt to reverse the metabolic abnormalities in PNP-deficient patients. It was hypothesized that toxic deoxyguanosine would follow its concentration gradient and exit from the patient's PNP-deficient lymphocytes through the serum and into the donated PNP-proficient erythrocytes, where it would be metabolized. Transfusion of normal erythrocytes did decrease nucleoside accumulation and increase uric acid production in PNP-deficient patients, followed by a transient increase in lymphocyte counts and a partial restoration of T-cell–dependent immunity (Rich et al., 1980; Staal et al., 1980a; Zegers et al., 1978). Disappointingly, immune reconstitution remained subtherapeutic and did not prevent devastating infections (Sakiyama et al., 1989). Moreover, the frequent erythrocyte transfusions were associated with iron overload and an increased risk of infections. The failure to restore T-cell immunity with erythrocyte transfusions could be due to inadequate and nonsustained PNP levels in target tissues or from the continuation of other metabolic consequences of PNP deficiency, such as depleted GTP production, in the cells.

Following the success of PEG-ADA in restoring immune function, a mutated form of *E. coli* PNP was covalently attached to PEG (Hershfield et al., 1991). PEG extended the activity of the bacterial PNP in vitro and reduced its immunogenicity in normal mice. Repeated PEG-PNP injections in PNP-deficient mice substantially increased whole-blood PNP activity to 20 percent of wild type, alleviated the secondary loss of dGK activity, and reduced urinary secretion of PNP substrates. PEG-PNP also reduced the concentration of dGTP in thymocytes of PNP-deficient mice, although not to normal levels. Treatment was followed by an increase in thymocyte numbers, improved lymphocyte responses to foreign antigens, and extended survival of the mice, even when housed in a nonsterile environment (Roifman and Grunebaum, unpublished). However, data indicating that PEG-ADA provides only partial and temporary immune reconstitution in ADA deficiency (Chan et al., 2005; Husain et al., 2007), possibly because the enzyme is not delivered to its native intracellular location, suggested that PEG-modified enzyme replacement may be suboptimal also for PNP deficiency. In addition, difficulties in production of PEG-PNP and the high cost of similar PEG-modified enzymes prompted us to evaluate alternative enzyme replacement therapy, such as PTD–PNP.

Protein transduction domains (PTDs), including the HIV TAT, are peptides capable of crossing biological membranes (Gump and Dowdy, 2007). PTDs deliver molecules attached to them into cells in a receptor-independent manner and cross the blood–brain barrier (Schwarze et al., 1999). Human PNP, fused with the 11 amino acids of TAT required for transcellular transport, maintained its enzyme activity. The PTD–PNP fusion protein was further improved and used in in vitro studies demonstrating that the PTD–PNP was able to rapidly enter PNP-deficient lymphocytes, increased PNP activity in cells for more than 96 hours, and was distributed in the cytoplasm, in a similar manner as endogenous PNP (Toro and Grunebaum, 2006). PTD–PNP administration increased PNP activity in all organs tested and urinary uric acid excretion in PNP-deficient mice. Twice-weekly injections of PTD–PNP corrected thymocyte development and T-lymphocyte function in PNP-deficient mice, improved weight gain, and significantly extended the survival of the mice, with no apparent toxicity (Toro and Grunebaum, 2006). Further preclinical studies are being conducted to establish the potential benefit of PTD–PNP for PNP-deficient patients.

Other therapeutic approaches have focused on directly improving immune function in PNP-deficient patients. A few patients have received fetal thymic transplants or thymic factors, but without significant benefit (Markert, 1991). In contrast, HSCT has been able to provide long-term immune reconstitution, albeit in only a few patients, and is currently considered the treatment of choice for PNP deficiency. The

initial review of HSCT in PNP deficiency was disappointing, with only one of five patients achieving immune reconstitution (Markert, 1991). However, subsequent reports have been more encouraging (Aytekin et al., 2008; Baguette et al., 2002; Carpenter et al., 1996 ; Classen et al., 2001; Delicou et al., 2007; Hallett et al., 1999; Isoyama et al., 2003; Myers et al., 2004). Bone marrow cells from HLA-matched family sibling donors were used in the majority of HSCTs for PNP deficiency (Aytekin et al., 2008; Baguette et al., 2002; Carpenter et al., 1996; Classen et al., 2001; Delicou et al., 2007). In addition, two PNP-deficient patients were transplanted with umbilical cord blood cells from unrelated donors. The first patient died 3 months after an HLA-matched cord blood transplant, which had been given subsequent to failing a previous transplant (Isoyama et al., 2003). The second patient received an umbilical cord blood transplantation following conditioning with busulfan, cyclophosphamide, and antithymocyte globulin (Myers et al., 2004). One year later, more than 98 percent of lymphoid and myeloid cells were of donor origin, with a significant improvement in the number of $CD4^+$ T lymphocytes and responses to mitogens. Difficulty in engrafting donor cells and graft rejection have been reported in several PNP-deficient patients, particularly after receiving T-cell–depleted haploidentical HSCT (Isoyama et al., 2003; Markert, 1991). Because most PNP-deficient patients have some residual T-cell immune function, it is not surprising that myeloablative conditioning, usually including cyclophosphamide and busulfan, was associated with improved engraftment (Carpenter et al., 1996; Rich et al., 1980; Staal et al., 1980a). However, the excessive vulnerability of PNP-deficient patients to chemotherapy (Blatt, 1990) together with the increased sensitivity of PNP-deficient cells to irradiation (Dror et al., 1993) prompted investigations into the use of less toxic conditioning regimens. One patient had normal T-cell numbers and adequate responses of T cells to antigens 1.5 years after an HLA-identical bone marrow transplant with busulfan and fludarabine conditioning (Classen et al., 2001). Another patient, who received bone marrow cells from an HLA-matched uncle with no conditioning, had 100 percent donor T cells 30 months after transplant and was able to overcome a disseminated BCG infection. B cells remained of recipient origin and the patient required intravenous immunoglobulin replacement (Aytekin et al., 2008). While these results are promising, the small number of patients assessed thus far prevents recommendation of an ideal conditioning regimen or stem-cell source for PNP deficiency.

The effects of HSCT on neurological outcome in PNP deficiency have been described in only a few patients, with just 1 to 4 years of follow-up. Some have reported that spastic paresis and tonus abnormalities remained static (Aytekin et al., 2008; Classen et al., 2001; Hallett et al., 1999), while ataxia, mobility, and cognitive function even improved (Aytekin et al., 2008; Classen et al., 2001; Myers et al., 2004; Singh, 2012), although the latter may also reflect improved overall medical status posttransplantation.

For the majority of PNP-deficient patients without genotypically HLA-identical donors, HCT with autologous cells that are corrected with the normal gene may have the benefit of avoiding the immunological complications of allogenic HCT. Retroviruses expressing murine PNP under the control of the murine leukemia virus were shown to increase PNP activity in T lymphocytes from PNP-deficient patients and partially correct the response of these cells to mitogens (Nelson et al., 1995). Recently we developed a lentivirus-based vector expressing human PNP under the control of the housekeeping human elongation factor 1 alpha (Liao et al., 2008). This lenti–PNP virus efficiently transduced lymphocytes from PNP-deficient patients as well as fibroblasts and bone marrow cells from PNP-deficient mice. Transplantation of nonlethally irradiated PNP-deficient mice with PNP-deficient bone marrow cells transduced with the lenti–PNP virus resulted in a transient increase of uric acid production, thymocyte maturation, and extended survival of the mice. However, 12 weeks after transplantation, PNP expression in corrected cells and the percentage of donor cells in the bone marrow of the mice decreased significantly (Liao et al., 2008). Optimizing the myeloablative conditioning given to recipients and ensuring long-term gene expression are among the factors expected to improve gene therapy for PNP-deficient patients.

In conclusion, the disappointing long-term prognosis for PNP-deficient patients treated with only supportive measures suggests that HCT should be offered to all patients with a genotypically HLA-identical or phenotypcally closely matched HLA donor. For the remaining patients, until effective enzyme or gene replacement therapies are available, the risks and benefits of transplantation using alternative sources of cells should be discussed.

ANIMAL MODELS

No naturally occurring animal models of ADA or PNP deficiency are known. However, targeted gene knockout mice have been generated to model both human conditions. An increasing amount of knowledge is being generated by studying these animals. It is worth noting, however, that while the knockout animals are obviously useful to direct the investigators' attention to relevant mechanistic and pathogenetic aspects of the conditions, the caveat exists that observations in mice may not immediately translate to the human diseases.

ADA Knockout Mice

At least two groups have succeeded in generating ADA knockout mice (Blackburn et al., 1995; Migchielsen et al., 1995, 1996; Wakamiya et al., 1995). However, the absence of ADA in mice leads to perinatal lethality. This is in contrast to the situation in humans, where individuals homozygous for deletion of the promoter and exon 1 have been born and presented with a disease indistinguishable from other patients with ADA-deficient SCID. Pathologically, newborn knockout mice exhibit marked hepatocellular degeneration, atelectasis, and intestinal cell death. In contrast to humans with ADA-deficient SCID, in whom marked lymphopenia is evident by 18 to 20 weeks of gestation, there were only minor reductions in $CD4^+/CD8^+$ lymphoid cells in livers of ADA knockout mice.

The problem of perinatal lethality was addressed with two different approaches. Expression of a human ADA transgene containing human regulatory elements rescued the mice (Migchielsen et al., 1996), demonstrating that the lethality was not due to disruption of a gene within the ADA gene. More significantly, however, mice could be rescued by expression of a transgene containing a promoter element restricting expression to the placenta and gastrointestinal tract (Blackburn et al., 1995). Normal mice have very high ADA activity in placenta and foregut, and the knockout mice were rescued by a minigene construct that selectively expressed ADA in these tissues. Several of the metabolic abnormalities seen in humans were reproduced in these mice, including increased deoxyadenosine and dAMP in the thymus and reduced SAH hydrolase in the thymus, spleen, and liver. The mice showed moderate reductions in size of the thymus and spleen and in absolute numbers of lymphocytes in these organs. In summary, evidence for a mild immunodeficiency was found. It is possible that the postnatal expression of ADA in intestinal tissues provided partial immunological rescue, suggesting that oral therapy might be ameliorative in human ADA deficiency. As an alternative explanation for the absence of profound immunodeficiency in the partially corrected mice, compensating pathways may exist in mouse that alter the pathophysiology of ADA deficiency. It is well recognized that the final common pathway for purine metabolism, ending in uric acid, is quite different in humans compared to virtually all other species. In addition, it is not infrequent that immunological knockouts give phenotypes that differ between man and mouse.

Filling the need for a better model of the human disease, a mouse deficient for ADA that does manifest combined immunodeficiency was then created by a two-stage genetic engineering strategy (Blackburn et al., 1998). These mice appear to reproduce not only the biochemical but also the immunological abnormalities of the human disease. This animal model may therefore help clarify the role of enzyme deficiency in production of various human phenotypic features. ADA-deficient splenic B lymphocytes show defects in proliferation and activation with a high propensity to undergo B-cell-receptor–mediated apoptosis. As a result, profound loss of germinal center architecture is noted, which may be responsible for impaired B-cell development (Aldrich et al., 2003). In addition, the finding of renal pathology in these animals suggests that the renal abnormalities previously reported in humans with ADA deficiency are a pleiotropic effect of ADA deficiency (Ratech et al., 1985). Treatment of these mice with PEG-ADA showed that while low doses could prevent the pulmonary insufficiency, the immunodeficiency could be ameliorated only by high doses of PEG-ADA, thus demonstrating a different impact of the therapy at the level of different tissues (Blackburn et al., 2000). Further, these mice provided evidence that the respiratory failure was attributable to IL-13–mediated accumulation of activated alveolar macrophages and eosinophils and mast cell degranulation with airway hyperresponsiveness (Blackburn et al., 2000, 2003; Chunn et al., 2001), thus offering insights into the mechanisms potentially responsible for asthmatic conditions and pulmonary insufficiency observed in some ADA-deficient humans. Furthermore, studies in these mice showed the development of lung and dermal fibrosis in association with increased levels of adenosine and key mediators of fibrosis, including transforming growth factor beta1, connective tissue growth factor, IL-13, and platelet-derived growth factors (Chunn et al., 2005, 2006; Fernandez et al., 2008). Treatment of ADA-deficient mice with the A(2A) receptor antagonist ZM-241385 prevented the development of dermal fibrosis in this model and confirmed a fibrogenic role for adenosine in the skin that may be speculated to contribute to the high incidence of dermatofibrosarcoma protuberans observed in ADA-deficient patients (Kesserwan et al., 2012). Finally, these mice have a specific bone phenotype characterized by alterations of structural properties and impaired mechanical competence as the result of decreased osteoclastogenesis and defective osteoblast function. The bone abnormalities observed in these ADA-deficient mice appear to have consequences on the bone marrow microenvironment and cause reduced in vitro and in vivo hematopoiesis (Sauer et al., 2009). These abnormalities may help explain the skeletal abnormalities characteristic of ADA-deficient humans and the myeloid dysplastic features recently observed in these patients.

PNP Knockout Mice

There are no known naturally occurring models for PNP deficiency. Several groups attempted to chemically induce transient PNP deficiency in dogs, rats, and mice; however, the metabolic and immune abnormalities differed from those observed in PNP-deficient patients (Bantia et al., 1996; Osborne and Barton, 1986; Osborne et al., 1986; Slichter et al., 1990). Subsequently, N-ethylnitrosourea mutagenesis was used to create three mice strains with different missense mutations in the PNP gene. PNP activity in the erythrocytes of these mice was severely reduced (1 to 4.6 percent) but not absent (Mably et al., 1989; Snyder et al., 1997). Progressive thymic hypocellularity due to a reduction of $CD4^+$ $CD8^+$ double-positive thymocytes was observed in these mice, which also showed a marked reduction in peripheral T-lymphocyte numbers and function. Although these mice shared some similarities with the human phenotype, the residual levels of PNP activities, the lack of dGTP accumulation in thymocytes, the delayed onset of the immune deficiency, and the normal lifespan of the mice were not representative of PNP-deficient patients (Snyder et al., 1997). Therefore, efforts were devoted to generate another PNP-deficient mouse model targeting the catalytic site of the murine PNP gene (Arpaia et al., 2000). These PNP-deficient mice share most of the metabolic, immune, and nonimmune abnormalities described in typical PNP-deficient patients, including very early death at 8 to 12 weeks of life (Table 14.10). PNP activity in the blood of PNP-deficient mice is less than 0.2 percent of normal, and their urine contains large amounts of the four PNP substrates (Arpaia et al., 2000). Uric acid production in PNP-deficient mice is diminished and the mice fail to gain weight (Toro and Grunebaum, 2006). At 3 weeks of age, the thymus of PNP-deficient mice is small and lacks proper corticomedullary distinction, with a marked reduction in total

thymocytes, primarily due to a reduced number of $CD4^+$ $CD8^+$ double-positive thymocytes, which have undergone accelerated apoptosis (Arpaia et al., 2000). The increased apoptosis of PNP-deficient double-positive thymocytes can be replicated ex vivo with low concentrations of deoxyguanosine, which induce significant apoptosis preferentially in the double-positive thymocyte population. Abnormal differentiation occurs also in the $CD4^-$ $CD8^-$ (double negative, DN) thymocytes, primarily among the $CD25^-$ $CD44^-$ DN4 population, while thymocyte proliferation, determined by BrDU incorporation, and T-cell-receptor V-beta chain rearrangements are unaffected (Papinazath et al., 2011). TRECs in the peripheral T lymphocytes of PNP-deficient mice are reduced, further indicating that PNP deficiency interferes with thymocyte development. In mitochondria isolated from PNP-deficient thymocytes, a marked increase in dGTP is found, which supports the hypothesis that accumulation of dGTP in the mitochondria of susceptible thymocytes is the basis for PNP immunodeficiency. Interestingly, dGK activity is reduced in PNP-deficient thymocytes, as well as all other tissues examined, which suggests that dGTP inhibits dGK (Arpaia et al., 2000). The number of peripheral T lymphocytes in PNP-deficient mice is markedly reduced, and these cells are unable to respond to mitogens or allogeneic stimulations. T lymphocytes from PNP-deficient mice are also more sensitive to ionizing irradiation and secrete reduced amounts of IL-2. As seen in many PNP-deficient patients, the B-cell compartment in PNP-deficient mice is less affected, with no changes in the frequency of mature B lymphocytes. All mice demonstrate a severely enlarged spleen, which contains reduced white pulp and T cells with increased numbers of inflammatory cells, including pre-B, myeloid, and plasma cells (Arpaia et al., 2000).

The frequent neurological defects seen in PNP-deficient patients led us to investigate for similar abnormalities in PNP-deficient mice. We found evidence of ataxia and abnormal motor coordination in the mice, together with changes in cerebellar Purkinje cells. These abnormalities are already evident in 2-week-old PNP-deficient mice, indicating that the neurological damage from PNP deficiency occurs early in life (Mansouri et al., 2012).

PNP-deficient mice also suffer from hepatomegaly, with significantly elevated bilirubin and liver transaminases, which has been reported in ADA-deficient patients and mice (Bollinger et al., 1996; Somech et al., 2009; Wakamiya et al., 1995) but not in PNP-deficient patients. Histological examination of the liver from PNP-deficient mice demonstrates marked hepatitis with distorted liver cells, inflammatory cell infiltrates (particularly plasma cells), and bile plugs (Grunebaum, unpublished). Despite extensive investigations, no infectious etiology could be detected.

In addition to revealing pathogenic mechanisms responsible for phenotypic abnormalities, PNP-deficient mice allow us to assess potential therapeutic options for PNP deficiency (Liao et al., 2008; Toro and Grunebaum, 2006). Among the different treatments studied extensively in our PNP-deficient mice, best results were obtained with PTD–PNP enzyme replacement, which corrected the metabolic and immune abnormalities as well as liver dysfunction, while prolonging survival for more than 1 year. Transplantations using normal bone marrow cells also corrected immune abnormalities, although liver manifestations continued, possibly because the metabolic improvement was only partial. Importantly, many PNP-deficient mice transplanted with normal cells lose the graft, suggesting that the survival advantage that PNP-proficient cells possess in a PNP-deficient environment may be inadequate.

Table 14.10 **COMPARISON BETWEEN PNP-DEFICIENT PATIENTS AND MURINE MODEL OF PNP DEFICIENCY**

	PNP-DEFICIENT PATIENTS	PNP-DEFICIENT MICE
Accumulating toxicity over time	Yes	Yes
Typical age of clinical symptoms	1–5 years	5–7 weeks
Deoxyguanosine and dGTP	Increased	Increased
Guanine and GTP	Decreased	Decreased
Uric acid production	Decreased	Decreased
$CD4^+$ T-lymphocyte numbers	Reduced	Reduced
T-lymphocyte responses	Reduced	Reduced
Hypoplastic thymus	Yes	Yes
Immune dysregulation	Yes	Yes
Large spleen	Occasional	Yes
Humoral immunity	Spared	Spared
Neurological abnormalities	Yes	Yes
Liver abnormalities	Not reported	Yes
Sensitivity to irradiation	Yes	Yes
Engraftment failure after transplant	Common	Common
Age of death without treatment	5–10 years	8–12 weeks

Transplanting gene-corrected PNP-deficient bone marrow cells into PNP-deficient mice was also associated with improved metabolic and immune functions, but these were transient and inferior to both enzyme replacement therapy and normal bone marrow transplantation. Interestingly, none of the therapeutic interventions corrected the neurological abnormalities in PNP-deficient mice, possibly because treatments were initiated at 3 to 4 weeks of age, after irreversible damage to the nervous system had already occurred. This emphasizes the importance of diagnosing and correcting PNP deficiency as early as possible.

Mouse models are undoubtedly providing valuable insights into the pathogenesis of immunodeficiency due to defects of purine nucleotide metabolism. However, important differences may exist between mice and humans regarding both critical metabolic networks and alternative biochemical pathways. Definitive conclusions on the significance of the findings observed in ADA and PNP knockout animals will therefore have to be supported by confirmatory results obtained from studies in human patients.

FUTURE DIRECTIONS AND CHALLENGES

Even though ADA deficiency and PNP deficiency have been known to be the molecular basis of immunodeficiency and have been extensively investigated for over two decades, major challenges remain. These range from diagnostics to new forms of therapy to insights as to basic biology that are yet to be obtained.

The discovery of the molecular basis of the various immunodeficiency disorders discussed in this volume makes it important to develop an overall strategy for diagnosing immune disorders. Currently, in a severely ill child, the first step is determining the presence or absence of ADA deficiency, since enzyme replacement therapy can provide a life-saving intervention. With the development of other therapeutic options for additional disorders, this commonly used strategy may well become outdated. With respect to diagnostics, ADA deficiency is an inherited disorder and therefore involves a family. The importance of experienced genetic counselors in helping family members to deal with issues of identification as carrier cannot be overemphasized. We are often contacted by family members to determine if they are carriers of the mutations(s) in their families. Currently, these investigations are "cottage industry" in approach. The development of "chip" technology for rapid scanning for mutations, now being applied on a research basis, could revolutionize rapid diagnosis. However, given the rarity of the disease, automated mutation detection is unlikely to be developed by industry. Should there be an "orphan drug" equivalent for diagnostics? Such an approach would greatly facilitate relatively noninvasive prenatal diagnosis not only for prevention but also for early institution of therapy when available. These concerns apply to virtually all of the immunodeficiency disorders.

With respect to basic biology, the role of ADA and adenosine is an area that is increasingly being investigated. Adenosine interacts with multiple adenosine receptors that have opposing actions in different cell types. ADA bound to CD26 at the cell surface plays a role in regulating local concentrations of adenosine. The potential role of adenosine in the clinical aspects of ADA deficiency has been ignored for some time. Based on the observation that children, after bone marrow transplant, were immunocompetent despite persistently elevated plasma adenosine concentrations, no major involvement of adenosine in the pathophysiology of immunodeficiency was assumed. However, adenosine appears to be important in the pathogenesis of asthma, which may be more common in ADA-deficient patients than in the general population, and has important roles in the skin and the cardiovascular and nervous systems. ADA also shows localization to the nervous system, and more recent in vitro experiments indicate a role for adenosine in regulating intracellular signaling pathways. One additional line of evidence of the importance of adenosine at the SNC level comes from the clinical observation that methotrexate-induced coma is caused by elevated levels of adenosine and can be resolved by administration of adenosine receptor antagonist (i.e., aminophylline). In addition, one of the most widespread clinical observations of physicians treating ADA-deficient children for immunodeficiency has been that alertness improves following enzyme replacement therapy. Insufficient data are available, however, in long-term survivors of ADA-deficient SCID after HSCT to rule out that these patients may show an intellectual or attention deficit.

With respect to therapy, the challenges are obviously high. A combined role for PEG–ADA and gene therapy could provide an alternative management option for ADA-deficient patients lacking suitable transplant donors, once the risks of gene transfer into the hematopoietic stem cells for this disease are carefully considered. As the clinical importance of adenosine receptor-mediated effects is clarified, interventions based on the use of receptor antagonist and/or inhibitors may be considered and developed.

Progress in the clinical management of PNP deficiency is acutely needed to modify what is currently a severe prognosis. The availability of effective enzyme replacement therapy for PNP deficiency, analogous to the use of PEG–ADA in ADA deficiency, is likely to change patient management, enabling temporary correction of the abnormal purine metabolism until definitive treatments such as allogeneic stem cell transplantation or autologous gene-corrected cells are available. Importantly, the effects of PNP deficiency on nonhematopoietic cells, such as neuronal cells, need to be determined, as this may lead to significant changes in understanding the pathogenic effects of PNP deficiency and choosing optimal treatments for patients. The availability of an animal model that closely reflects the human disease is critical for all of these goals. Finally, some of the toxic effects of PNP deficiency may not be reversible with treatment, emphasizing the need for early recognition of affected patients through newborn screening and an increased awareness among health care providers.

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15.

SCID DUE TO DEFECTS IN T-CELL-RECEPTOR-ASSOCIATED PROTEIN KINASES (ZAP-70 AND LCK)

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ZAP-70 is a ζ-chain-associated protein of 70,000 kDa (MIM + 176947). Deficiency of ZAP-70 leads to a rare autosomal recessive form of severe combined immunodeficiency (SCID) characterized by the selective absence of $CD8^+$ T cells and abundant $CD4^+$ T cells in the peripheral blood that are unresponsive to T-cell-receptor (TCR)-mediated stimuli in vitro. Peripheral T lymphocytes from most affected patients demonstrate defective signaling following TCR engagement by antigen because of inherited mutations within the kinase domain of the cytoplasmic protein tyrosine kinase (PTK) ZAP-70. ZAP-70 deficiency provided the first evidence that PTKs, and ZAP-70 in particular, are required for normal human T-cell development and function. Deficiency of another critical PTK, Lck, has been associated with a SCID phenotype in isolated patients as well.

T-CELL SIGNAL TRANSDUCTION

The processes involved in CD4 versus CD8 selection during T-cell ontogeny are known to require intact signal transduction through the TCR of differentiating thymocytes (Cheng and Chan, 1997; Palacios and Weiss, 2004, 2007; Pfeffer and Mak, 1994; Robey and Fowlkes, 1994;). The signal transduction events mediated by the TCR on thymocytes are similar to those responsible for the activation of mature T cells. Propagation of antigen-binding signals from the TCR to the nucleus is primarily dependent on the activation of the PTKs Lck and ZAP-70 (reviewed in Cheng and Chan, 1997; Palacios and Weiss, 2004, 2007; Wange and Samelson, 1996; Weiss and Littman, 1994). PTK activation is a rapid and critical event that results in phosphorylation of downstream proteins, including phospholipase Cγ1 (PLCγ1), linker for activation of T cells (LAT) and Src homology 2 (SH2) domain-containing 76 kDa leukocyte protein (SLP-76; reviewed in Palacios and Weiss, 2004, 2007; Rudd, 1999; Wange and Samelson, 1996). These tyrosine phosphorylation events are critical for the recruitment of numerous other signaling molecules required for the mobilization of intracellular free calcium ($[Ca^{2+}]_i$) and activation of downstream pathways that culminate in the activation of T cells and initiation of T-cell-specific responses, described in more detail in Chapter 6.

T-CELL-RECEPTOR-ASSOCIATED PROTEIN TYROSINE KINASES

Stimulation of the TCR results in activation of certain nonreceptor cytoplasmic PTKs whose functions are critical for lymphocyte responses to antigens (see Chapter 6). The importance of these PTKs to T-cell development and function has been established by findings of immunodeficiency in both humans and PTK-deficient mice. Of the PTKs that have been described in T cells, the function of Lck is best understood. Lck is a 56 kDa Src family PTK that is expressed at high levels in peripheral T cells and in thymocytes at all stages of maturation (Perlmutter et al., 1988). Numerous in vivo and in vitro studies have confirmed the importance of Lck to normal T-cell signal transduction. Recruitment of ZAP-70 to the TCR is primarily dependent on the phosphorylation of consensus immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3 ε and TCRζ chains by Lck (Au-Yeung et al., 2009; Gauen et al., 1994; Iwashima et al., 1994; Weil et al., 1995; Weiss and Littman, 1994). The ITAMs are found either singly or multiply (3 copies) in the CD3ε and TCR ζ chains, respectively, and mediate TCR interactions with PTKs involved in signal transduction and with ZAP-70 in particular (Gauen et al., 1994; Irving et al., 1993; Weiss and Littman,

1994). Lck is also primarily responsible for mediating ZAP-70 phosphorylation, thereby triggering critical downstream signaling events required for T-cell activation (Au-Yeung et al., 2009; Chan et al., 1995; Watts et al., 1994).

Studies in genetically altered mice demonstrate that Lck is required for differentiation events in the transition of double-negative (DN; pre-TCR$^+$ CD4$^-$ CD8$^-$) thymocytes to double-positive (DP; TCR$^+$ CD4$^+$ CD8$^+$) thymocytes (Levin et al., 1993; Molina et al., 1992;). Disruption of Lck results in a SCID phenotype characterized by markedly decreased DP thymocytes and few single-positive (SP; CD4$^+$ or CD8$^+$) thymocytes or peripheral T cells (Molina et al., 1992). This block in T-cell development is related to a requirement for Lck in signaling through the pre-TCR CD3 complex (Levelt et al., 1995; Mombaerts et al., 1994; Palacios and Weiss, 2004). Lck is expressed prior to TCRβ chain rearrangement, and its activation induces thymocyte proliferation (between the DN and DP stages); moreover, it initiates TCRα chain rearrangement and allelic exclusion of TCRβ chain gene rearrangement (Anderson et al., 1993). ZAP-70 is a 70 kDa Syk family PTK expressed exclusively in all thymocyte subpopulations, peripheral T cells, natural killer (NK) cells, and aberrantly in neoplastic CD19$^+$ CD5$^+$ CD23$^+$ B cells in a subpopulation of patients with poor-prognosis B-cell chronic lymphocytic leukemia (Chan et al., 1992; Chiorazzi et al., 2005; Palacios and Weiss, 2007). Recruitment of ZAP-70 to the TCR is essential for its subsequent activation and for induction of downstream signaling events critical for induction of T-cell-specific responses (Au-Yeung et al., 2009; Wange et al., 1995b; Weil et al., 1995; Weiss and Littman, 1994). As described above, Lck mediates the phosphorylation of both tyrosine residues within each ITAM of TCRζ and CD3ε that occurs prior to ZAP-70 recruitment to the TCR (see Fig.6.2 in Chapter 6) (Gauen et al., 1994; Iwashima et al., 1994; Straus and Weiss, 1992; van Oers and Weiss, 1995; Weil et al., 1995). High-affinity binding of ZAP-70 to ζ and CD3ε requires the cooperative binding of both ZAP-70 SH2 domains (Au-Yeung et al., 2009; Gauen et al., 1994; Hatada et al., 1993; Isakov et al., 1995; Iwashima et al., 1994; Wange et al., 1993). Although association of ZAP-70 with the phosphorylated ITAM-containing subunits of the TCR releases ZAP-70 from its autoinhibited conformation, binding to the TCR alone is insufficient to induce ZAP-70 kinase activity (Au-Yeung et al., 2009; Madrenas et al., 1995; van Oers et al., 1994). ZAP-70 itself must be phosphorylated at specific tyrosine residues to increase its catalytic function (Chan et al., 1995; Neumeister et al., 1995; Wange et al., 1995a, 1995b; Watts et al., 1994). Tyrosine phosphorylation of ZAP-70 is mediated by Src family PTKs and by autophosphorylation activity (Au-Yeung et al., 2009; Chan et al., 1995; Neumeister et al., 1995; Wange et al., 1995a; Watts et al., 1994). Much of the increase in ZAP-70 catalytic function following TCR stimulation is associated with specific phosphorylation of tyrosine residue 493 by Lck or ZAP-70 itself (Au-Yeung et al., 2009; Chan et al., 1995; Watts et al., 1994). However, ZAP-70 enzymatic activity is also negatively regulated by phosphorylation of the adjacent tyrosine residue 492 (Chan et al., 1995; Wange et al., 1995a; Zhao and Weiss, 1996). Additional tyrosine residues within interdomain B, a region bridging the SH2 and kinase domains of ZAP-70, are phosphorylated and function as positive regulators of TCR signaling; these include tyrosine 315 (the binding site for the Vav guanine nucleotide exchange factor and CT10 regulator of kinase II, CrkII) and tyrosine 319, which is a binding site for PLCγ1 and Lck (Au-Yeung et al., 2009; Di Bartolo et al., 1999; Gelkup et al., 2005; Gong et al., 2001; Zhao and Weiss, 1999; Wu et al., 1997; Williams et al., 1999). Tyrosines 315 and 319 may also participate in regulation of ZAP-70 catalytic activity via an autoinhibitory switch mechanism (Au-Yeung et al., 2009). In contrast, phosphorylation of tyrosine 292 within interdomain B of the ZAP-70 protein inhibits TCR signaling by serving as a binding site for the E3 ubiquitin ligase c-Cbl, which targets ZAP-70 for ubiquitination and degradation (Lupher et al., 1996; Meng et al., 1999; Rao et al., 2000; Wang et al., 2000).

Following ZAP-70 phosphorylation and activation, multiple downstream signaling events occur that result in T-cell activation. Phosphorylated ZAP-70 triggers recruitment of other molecules to the signaling complex, resulting in transduction of the TCR-mediated signal. Specific in vivo substrates for ZAP-70 include LAT and SLP-76 (Wardenburg et al., 1996; Williams et al., 1998, 1999; Zhang et al., 1998). Recruitment to the signaling complex is mediated by the SH2 domains contained within each protein. Additional SH2 domain-containing signaling molecules recruited to the TCR via interactions with ZAP-70 or ZAP-70 substrates include c-Cbl, PLCγ1, CrkII, Vav, and the p85 subunit of PI3 kinase (Au-Yeung et al., 2009; Duplay et al., 1994; Finco et al., 1998; Katzav et al., 1994; Neumeister et al., 1995; Raab et al., 1997; Straus et al., 1996; Wardenburg et al., 1996; Wu et al., 1997; Yablonski et al., 1998). Formation of these protein complexes at the plasma membrane results in signal amplification and culminates in the transcription of genes involved in T-cell activation.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF ZAP-70 DEFICIENCY

Eight children from five families were originally described with SCID due to ZAP-70 deficiency (Arpaia et al., 1994; Chan et al., 1994a; Elder et al., 1994; Gelfand et al., 1995). These patients included six children from three genetically isolated Mennonite kindreds and two children from separate, unrelated (Hispanic and Caucasian) families, who were products of consanguineous relationships. Similar to other forms of SCID, each affected child presented within the first 2 years of life with a history of recurrent infections and failure to thrive (Table 15.1) (Elder et al., 1995; Gelfand et al., 1995; Monafo et al., 1992; Roifman et al., 1989). Seven of these children did well following allogeneic bone marrow transplantation (BMT); one child of Mennonite background died 2 months after BMT (Monafo et al., 1992). Additional patients with ZAP-70 deficiency have since been described and have had similar clinical presentations (Elder et al., 2001; Matsuda et al., 1999; Meinl et al., 2000; Noraz et al., 2000; Toyabe et al., 2001; Turul et al., 2009).

Table 15.1 CLINICAL FINDINGS IN EIGHT PATIENTS WITH ZAP-70 DEFICIENCY

FINDINGS	NUMBER OF AFFECTED CHILDREN
Infections	
Bacterial (otitis media, pneumonia)	**4**
P. jiroveci pneumonia	3
Cytomegalovirus retinitis or other infection	**2**
Parainfluenza pneumonitis	**1**
Chronic diarrhea or rotaviral enteritis	**6**
Severe or disseminated varicella	**1**
Oral or cutaneous candidiasis	**3**
Failure to thrive	**4**
Physical Findings	
Presence of lymph tissue	**5**
Hepatosplenomegaly	**1**
Chronic eczematoid rash	**2**
Thymic shadow on chest X-ray	**8**

Most ZAP-70–deficient patients have detectable lymphoid tissue and normal or elevated peripheral blood lymphocyte counts. Thymic histology in ZAP-70 deficiency is remarkable for normal percentages of DP and SP $CD4^+$ thymocytes, although there are very few, if any, SP $CD8^+$ cells (Gelfand et al., 1995; Roifman et al., 1989). With regards to the actual structure of the thymus, the formation of the thymic medulla appears to be defective (Negishi et al., 1995).

LABORATORY FINDINGS IN ZAP-70 DEFICIENCY

All patients have normal to elevated numbers of circulating lymphocytes (Table 15.2). Phenotypic analysis of their peripheral blood lymphocytes by flow cytometry reveals normal or increased percentages of $CD3^+$ (38 to 80 percent) and $CD4^+$ T cells (37 to 75 percent) because of the lack of $CD8^+$ T cells (0 to 3 percent) (Elder et al., 1995, 2001; Gelfand et al., 1995; Matsuda et al., 1999; Monafo et al., 1992; Noraz et al., 2000; Roifman et al., 1989). Expression of T-cell surface proteins other than CD8 is normal. In those patients in whom human leukocyte antigen (HLA) typing and Southern blot analysis have been performed, peripheral blood T cells were demonstrated to be polyclonal and not maternal in origin (Arpaia et al., 1994; Elder et al., 1995). Absent proliferative responses to alloantigen and to a variety of mitogens in vitro, including phytohemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A, *Candida albicans*, and anti-CD3 monoclonal antibody (MAb), are noted on testing of patient lymphocytes, confirming the diagnosis of SCID. Failure to reject an allogeneic skin graft was demonstrated in one ZAP-70–deficient patient (Roifman et al., 1989). NK-cell numbers and activity are relatively normal (Elder et al., 1994). All patients have normal numbers of B cells, but some differences in B-cell function have been noted between patients. Normal serum immunoglobulins and specific antibody production have been demonstrated in a minority of patients (Roifman et al., 1989), but most patients have hypogammaglobulinemia and lack specific antibody production, necessitating monthly intravenous immunoglobulin administration (Elder et al., 1995, 2001; Gelfand et al., 1995). One child had IgE reactive with food allergens but did not demonstrate atopy or intestinal symptoms related to food ingestion (Toyabe et al., 2001).

In the original eight patients with ZAP-70 deficiency, the markedly abnormal distribution of peripheral T-cell subsets and the absence of T-cell proliferation to any stimulus transduced by the TCR led to studies that documented defects within the proximal TCR signaling pathway, later proven to be due to absence of ZAP-70 function. The patients' T lymphocytes exhibited diminished or absent $[Ca^{2+}]_i$ mobilization after stimulation with anti-CD3 MAb (Arpaia et al., 1994; Chan et al., 1994a; Elder et al., 2001; Gelfand et al., 1995). Furthermore, induction of most cytoplasmic tyrosine phosphoproteins was defective after co–cross-linking of the TCR on patient T cells with either anti-CD3 or a combination of anti-CD4 and anti-CD3 MAbs (Arpaia et al., 1994; Elder et al., 1995, 2001). In contrast to their absence of cell proliferation to TCR-mediated stimuli, ZAP-70–deficient lymphocytes proliferate normally to phorbol myristic acetate (PMA) plus ionomycin, agents that bypass proximal TCR signaling events by mimicking actions of the second messengers Ras and $[Ca^{2+}]_i$, respectively. In addition, interleukin (IL)-2 production is detected when lymphocytes are stimulated with PMA plus ionomycin, in contrast to the lack of detectable IL-2 production when T cells are incubated with anti-CD3 or PHA (Arpaia et al., 1994; Elder et al., 1995).

MOLECULAR BASIS OF ZAP-70 DEFICIENCY

In most affected patients, ZAP-70 protein is not detected by immunoblot (Arpaia et al., 1994; Chan et al., 1994a; Elder et al., 1994; Gelfand et al., 1995; Matsuda et al., 1999; Noraz et al., 2000; Toyabe et al., 2001). In contrast, normal levels of other PTKs are present in patient T cells. Additional studies in one patient demonstrated that Lck function was normal: it could be coprecipitated with CD4, was inducibly phosphorylated in response to cross-linking of surface CD3 and CD4, and exhibited normal autophosphorylation activity in vitro (Elder et al., 1995). One child with SCID had undetectable ZAP-70 mRNA, but his underlying mutation(s) were not identified (Gelfand et al., 1995). The majority of patients have normal levels of full-length ZAP-70 mRNA as compared to controls.

At least 16 separate mutations in the gene encoding ZAP-70 (gene symbol *ZAP70* or *SRK*, Syk-related tyrosine kinase) have been identified in affected children (Arpaia et al.,

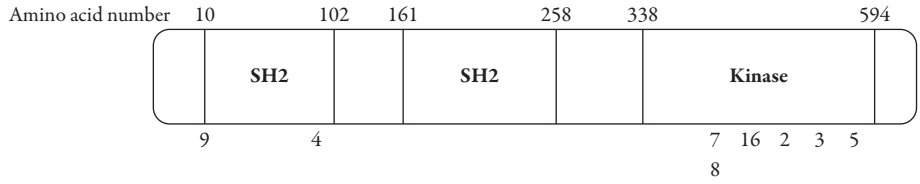
Table 15.2 LABORATORY FINDINGS IN PATIENTS WITH ZAP-70 DEFICIENCY

TESTS	RANGE OF PATIENT VALUES	NORMAL RANGE
Lymphocyte Phenotype		
Absolute lymphocyte count	4.0–20.0 × 10^9/L	3.5–8.0 × 10^9/L
$CD3^+$ cells	35–80%	50–85%
$CD4^+$ cells	35–75%	25–70%
$CD8^+$ cells	0–10%	12–40%
$CD3^+$ $CD8^+$ cells	0–3%	10–40%
TCRα/β^- cells	55–80%	50–85%
TCRγ/δ^- cells	0.2–?%	1–10%
$CD3^+$ $CD69^+$ cells	0–3%	25–55%
$CD16^+$ $CD56^+$ cells	5–15%	5–30%
$CD19^+$ cells	13–40%	6–25%
Serum Immunoglobulin Levels		
IgG (pre-IVIG)	40–1,250 mg/dL	350–1,250 mg/dL
IgA	40–160 mg/dL	10–50 mg/dL
IgM	65–170 mg/dL	40–200 mg/dL
Isohemagglutinins	0–<1:1 titer	0–1:128 titer
Specific antibody production	1 pt (levels not reported)	
Lymphocyte Proliferative Function		
Mitogens (PHA, PWM)	0–1,100 cpm	35,000–230,000 cpm
Soluble antigen (tetanus, candida)	0–750 cpm	1,500–100,000 cpm
Alloantigen (MLC)	125–5,000 cpm	10,000–150,000 cpm
Anti-CD3	200–1,000 cpm	20,000–95,000 cpm
IL-2	1,000–20,000 cpm	1,000–6,000 cpm
PMA	15,000–50,000 cpm	1,500–110,000 cpm
PMA plus ionomycin	40,000–200,000 cpm	25,000–170,000 cpm
PMA plus IL-2	100,000–150,000 cpm	50,000–120,000 cpm
SAC (B-cell mitogen)	5,000–25,000 cpm	5,000–25,000 cpm
IL-2 Production		
PHA, anti-CD3	none	
PMA plus ionomycin	normal	
NK cell cytotoxicity	20–30%	20–50%

1994; Chan et al., 1994a; Elder et al., 1994, 2001; Matsuda et al., 1999; Meinl et al., 2000; Noraz et al., 2000; Picard et al., 2009; Toyabe et al., 2001; Turul et al., 2009; and E. Faria, J. Chinen, M. Hoenig, I. Barlan, personal communications). Most mutations occur within the kinase domain of the ZAP-70 protein and significantly affect both protein stability and catalytic activity (Fig. 15.1).

ZAP-70 MUTATION ANALYSIS

Pedigree analyses have confirmed that ZAP-70 deficiency is an autosomal recessive genetic syndrome and that affected children have failed to inherit a normal allele of the ZAP-70 gene located on human chromosome 2q12 (Chan et al., 1994a). The molecular defects reported to date are listed in Figure 15.1. All six children from the three genetically related Mennonite families inherited at least one mutant ZAP-70 allele with a splicing error due to a G-to-A transition within an intron, generating a new splice acceptor site upstream of the normal splice site. Specifically, three children from two families were homozygous for a 9 bp insertion (CTTGAGCAG) into the coding sequence after nucleotide 1832. This mutation results in the addition of three amino acid residues, leucine, glutamic acid, and glutamine (LEQ), within the ZAP-70 catalytic domain following residue 541 (K541insLEQ)



Mutant allele	DNA Mutation	Consequence to Protein
1	13 bp deletion (nts 1719–1731)	Deletion/frameshift/premature termination after 35 codons (K504_P508delfs×35)
2	C to A transition at nt 1763	Missense (S518R)
3	G to A transition at nt 1833	Splicing error (K541_K542insLEQ)
4	C to A transition at nt 448	Missense (P80Q)
5	A to T transition at nt 1923	Missense (M572L)
6	C to T at nt 1729	Missense (A507V)
7	C to T at nt 1602	Missense (R465C)
8	G to A at nt 1603	Missense (R465H)
9	? (no ZAP-70 mRNA)	

Figure 15.1 Schematic depiction of wild-type ZAP-70 protein and the identified mutant alleles. GenBank accession number is L05148 for the human AP-70 cDNA sequence.

(Arpaia et al., 1994). Three children from the remaining Mennonite family inherited this mutant allele along with a second mutant allele and were thus compound heterozygotes for two ZAP-70 mutations (Chan et al., 1994a). The second mutation in these Mennonite cases is a C-to-A transition at position 1763, which results in a serine-to-arginine substitution at amino acid 518 (S518R) within a highly conserved region of all Syk family PTK catalytic domains.

In a fourth affected and unrelated family, a single patient with ZAP-70 deficiency was homozygous for a 13bp deletion at nucleotides 1719 to 1731 (Elder et al., 1994). This deletion results in a translational frameshift after residue 503 and introduces a premature stop 35 codons downstream, yielding a mutant protein truncated by 82 amino acids (K504_P508delfsX35). One Japanese patient with CD8 deficiency was a compound heterozygote for temperature-sensitive mutations in ZAP-70: a C-to-A transition at position 448 resulting in a proline-to-glutamine substitution at residue 80 (P80Q), and an A-to-T transition at position 1923 that substitutes a leucine for methionine at amino acid 572 (M572L) within the ZAP-70 protein (Matsuda et al., 1999). Homozygous mutations resulting in Leu337Arg and Cys564Arg substitutions have recently been described in two other patients (Turul et al., 2009).

Three infants with SCID inherited a homozygous missense mutation within the highly conserved DLAARN motif in the ZAP-70 kinase domain (Elder et al., 2001; Toyabe et al., 2001). In two children, the mutation was a C-to-T transition at bp 1602, which results in an arginine-to-cysteine conversion at residue 465 (R465C). Unlike other described ZAP-70 mutations, this mutation only modestly affects protein stability, but it abrogates ZAP-70 catalytic function. Moreover, R465C is comparable to the mutation (R464C) that spontaneously arose in the inbred Strange (ST) mouse colony (Wiest et al., 1997). In contrast to ST mice, which lack any SP thymocytes and peripheral T cells, these patient had typical findings of ZAP-70 deficiency and lacked only circulating $CD8^+$ T lymphocytes (Elder et al., 2001). This case provides the first description of identical mutations in ZAP-70 having distinct T-cell developmental consequences in humans and mice. Subsequently, an unrelated patient was determined to be homozygous for a G-to-A transition at position 1603, resulting in an arginine-to-histidine (R465H) conversion at the same critical residue within the DLAARN motif (Toyabe et al., 2001). However, this latter patient lacked ZAP-70 protein expression.

Interestingly, identical ZAP-70 mutations can apparently result in phenotypic heterogeneity; an Ala507His substitution in the ZAP-70 kinase domain was found to result in the absence of ZAP-70 protein expression and $CD8^+$ T cells in two siblings and an apparently unrelated third patient (Noraz et al., 2000; Turul et al., 2009). While the two siblings had a typical SCID phenotype necessitating hematopoietic stem cell transplantation (HSCT; Noraz et al., 2000), the third had a mild clinical phenotype until the age of 3 years, when she was diagnosed with a B-cell lymphoma (Turul et al., 2009, and O. Sanal, personal communication). This significant clinical variability is reminiscent of that observed in CD3γ deficiency (Recio et al., 2007), and while not understood in either disease, likely reflects the involvement of other genetic and/or environmental factors.

In the published literature, all identified mutations, with the exception of the R465C mutation, significantly decrease the stability of ZAP-70 protein in vitro (Chan et al., 1994a; Elder et al., 1994; Matsuda et al., 1999; Noraz et al., 2000). Furthermore, most mutations that change the sequence of the kinase domain of ZAP-70 affect its enzymatic activity. However, newly described mutations in the SH2 domain affect ZAP-70 function without interfering with protein stability (Chinen and Taylor, unpublished observations). In vitro kinase assays have confirmed that mutant forms of ZAP-70 are catalytically inactive (Chan et al., 1994a; Elder et al., 1994, 2001; Noraz et al., 2000).

FUNCTIONAL ASPECTS OF ZAP-70 DEFICIENCY

The phenotype of ZAP-70 deficiency in humans presents a notable paradox: although a single signaling pathway had previously been assumed to be required for both thymic selection and peripheral T-cell activation, the abundant $CD4^+$ T cells in this disorder, which presumably were selected positively in the thymus, are refractory to TCR-mediated activation in the periphery. Thymic tissue from three ZAP-70–deficient patients revealed mostly DP thymocytes and normal numbers of SP $CD4^+$ cells, but very few if any SP $CD8^+$ thymocytes (Gelfand et al., 1995; Roifman et al., 1989).

ZAP-70 has been shown in mice to be specifically required for normal progression of DN thymocytes to DP and SP cells (Chu et al., 1999; Palacios and Weiss, 2007). However, results in ZAP-70–deficient patients confirm that ZAP-70 is critical for CD8 selection and peripheral $CD4^+$ and $CD8^+$ T-cell signaling but is dispensable for CD4 selection in the thymus in humans. An explanation for the presence of normal numbers of $CD4^+$ T cells is that the ZAP-70–related PTK Syk, which is expressed at higher levels in the thymus than in the periphery and is required for initial pre-TCR signaling, is able to rescue CD4 selection in the absence of ZAP-70 (Au-Yeung et al., 2009; Chan et al., 1994b; Gelfand et al., 1995; Palacios and Weiss, 2007). A role for Syk in pre-TCR signaling during thymocyte selection is supported by findings that DP thymocytes from a ZAP-70–deficient patient were able to mobilize $[Ca^{2+}]_i$ upon CD3 cross-linking (Gelfand et al., 1995). Comparable induction of tyrosine phosphoproteins was seen in a human T-cell leukemia virus (HTLV)-1-transformed $CD4^+$ thymocyte line. ZAP-70–deficient thymocytes demonstrated higher basal Syk expression and increased Syk phosphorylation after TCR stimulation than control HTLV-1-transformed thymocytes. Increased Syk activity in ZAP-70–deficient thymocytes may thus compensate at least in part for the lack of ZAP-70 function. In support of this finding, downregulation of Syk expression during thymocyte differentiation occurs later in humans than in mice (Au-Yeung et al., 2009; Cheng and Chan, 1997; Chu et al., 1999).

Although the differential ability of Syk to replace ZAP-70 in CD4 versus CD8 selection events remains unclear, CD4 lineage cells develop in the absence of ZAP-70 function. Moreover, Syk activity, which is downregulated in mature T cells, cannot compensate for the loss of ZAP-70 function that is required for peripheral $CD4^+$ T-cell signal transduction in affected patients (Au-Yeung et al., 2009; Chu et al., 1999; Siggs et al., 2007). Peripheral T cells from some ZAP-70–deficient patients have been shown to express high levels of Syk after long-term culture and under these specific conditions are capable of partial TCR signaling (Noraz et al., 2000). However, findings have been more variable in freshly isolated ZAP-70–deficient T cells and suggest that survival of these cells does not require ZAP-70–mediated TCR signaling or upregulation of Syk expression (Elder et al., 2001; Toyabe et al., 2001) and likely depends on cytokine signals such as those mediated by IL-7. Further studies are needed to characterize how the absence of ZAP-70 and expression of the related Syk PTK may modulate the antigen specificity and TCR repertoire of $CD4^+$ T cells differentiating in patients with ZAP-70 deficiency.

ZAP-70 AND AUTOIMMUNITY

Recent studies in mice have shown that decreases in TCR signaling, caused by point mutations in the ZAP-70 gene, can alter the TCR repertoire of mature T cells and result in autoimmunity (Hirota et al., 2007; Jakob et al., 2008; Sakaguchi et al., 2003; Siggs et al., 2007). Indeed, two independently performed screens for cellular immunodeficiencies in mice treated with the N-ethyl-N-nitrosourea mutagen revealed the causative affected gene to be ZAP-70 (Jakob et al., 2008; Siggs et al., 2007). In both models, hypomorphic mutations in the ZAP-70 kinase domain (homozygous W163G and composite I368F/W504R substitutions) resulted in the excessive production of autoantibodies and immunoglobulin E, likely due to defects in negative selection (Jakob et al., 2008; Siggs et al., 2007). Moreover, an independent spontaneous hypomorphic mutation in the SH2 domain of ZAP-70 (W163C) was shown to result in a chronic arthritis, and in this latter case, the self reactive $CD4^+$ T cells have been found to preferentially differentiate into IL-17–secreting Th17 cells (Hirota et al., 2007; Sakaguchi et al., 2003). Thus, hypomorphic mutations in ZAP-70 can alter TCR signaling capacities, compromising immunological tolerance.

Notably, the first patient with a hypomorphic ZAP-70 mutation has recently been reported (Picard et al., 2009). In this case, a homozygous G-to-A transition in intron 7 of the genomic ZAP-70 DNA (836+121) resulted in an 80 percent decrease in ZAP-70 protein in a 9-year-old patient with decreased $CD4^+$ and $CD8^+$ T cells. The abnormally spliced ZAP-70 product appears to be rapidly degraded and the low level of detected protein was the wild-type splice form. The clinical consequences in this patient were attenuated as compared to patients totally lacking ZAP-70 protein, and significantly, this patient did not shown signs of autoimmunity. It is nonetheless important to note that in contrast with mice suffering from ZAP-70–associated autoimmunity, the ZAP-70 protein expressed in this patient's T cells was wild type. Other patients with defects in ZAP-70 have presented with eczematous lesions, skin infiltrates, and manifestations of ulcerative colitis, which may be due to autoimmunity (Katamura et al., 1999; Parry et al., 1996). As such, it is likely that ZAP-70–associated defects will be found to result in autoimmune symptoms in patients, but confirmation will require more detailed characterization of patients with hypomorphic ZAP-70 mutations.

STRATEGIES FOR DIAGNOSIS OF ZAP-70 DEFICIENCY

Patients with SCID characterized by variable hypogammaglobulinemia, relatively normal numbers of peripheral blood lymphocytes, normal or only modestly decreased T cells, but notable absence of $CD8^+$ T cells (<5 percent) should

be screened for ZAP-70 deficiency. The diagnosis would be suggested by lack of T-cell proliferation in vitro to TCR stimuli, such as PHA and anti-CD3 MAb, but proliferation to PMA plus ionomycin comparable to that of normal T cells. Further studies to confirm a proximal TCR signaling defect include defective $[Ca^{2+}]_i$ mobilization and poor tyrosine phosphoprotein induction after stimulation through the TCR. In most cases, blotting for ZAP-70 protein demonstrates lack of ZAP-70 protein expression. If phosphotyrosine induction is abnormal but ZAP-70 protein is present, cDNA or genomic DNA sequencing will be required to characterize the mutation(s).

CARRIER DETECTION AND PRENATAL DIAGNOSIS

If the ZAP-70 mutations are known in an affected proband, then both carrier detection in relatives and prenatal diagnosis of an at-risk fetus can be performed. Individuals heterozygous for a mutant ZAP-70 allele are immunologically normal. Therefore, molecular techniques would be required to establish heterozygosity. However, since ZAP-70 deficiency is extremely rare and has primarily been demonstrated in consanguineous and genetically inbred families, carrier detection is useful for only a very few individuals in isolated population groups.

TREATMENT AND PROGNOSIS

Patients with ZAP-70 deficiency require HSCT for cure of SCID. Six of the original ZAP-70–deficient patients who were successfully transplanted received one of the following regimens: (1) histocompatible BMT from a sibling after conditioning with busulfan and cytoxan (BuCy) and antithymocyte globulin (ATG) (one child; A. H. Filipovich, personal communication); (2) T-cell–depleted BMT from a parent either without prior conditioning (one child; E. W. Gelfand, personal communication) or after cytoxan/ATG conditioning (one child; Elder et al., 1995) or after BuCy/ATG conditioning (one child; A. H. Filipovich, personal communication); (3) HLA-matched unrelated BMT after BuCy/ATG conditioning (one child; A. H. Filipovich, personal communication); or (4) partial HLA-matched unrelated BMT after BuCy/ATG conditioning (one child; A. H. Filipovich, personal communication). Mobilized peripheral blood stem cells from a parent have also been used with variable success to reconstitute ZAP-70–deficient children (Elder et al., 2001; Skoda-Smith et al., 2001; Toyabe et al., 2001). In one case, a paternal mobilized peripheral blood stem cell transplant followed by treatment with anti-CD20 MAb (Rituximab) successfully reconstituted a ZAP-70–deficient child who developed Epstein-Barr virus (EBV)-negative non-Hodgkin's B-cell lymphoma following an attempted maternal T-cell–depleted haploidentical BMT (Skoda-Smith et al., 2001). Monthly intravenous gammaglobulin therapy is often required for some time following HSCT.

ANIMAL MODEL OF ZAP-70 DEFICIENCY

Genetically altered mice lacking the entire ZAP-70 locus exhibit markedly defective T-cell activation and development (Negishi et al., 1995). In contrast to findings in ZAP-70–deficient patients, T-cell ontogeny in ZAP-70 knockout mice is blocked at the DP stage of thymocyte differentiation. Thymocytes from mutant mice fail to mobilize $[Ca^{2+}]_i$ after TCR cross-linking by anti-CD3 mAb, and neither SP $CD4^+$ nor $CD8^+$ thymocytes develop, and peripheral T cells are absent. These findings, as well as the demonstration that SP $CD4^+$ and $CD8^+$ T-cell development could be rescued by expression of a functional human ZAP-70 cDNA in mutant thymocytes, suggest that ZAP-70 is required for selection of both major thymocyte subsets in mice (Negishi et al., 1995; Palacios and Weiss, 2007). A comparable lymphocyte phenotype is seen in ST mice, which develop SCID due to a spontaneously arising mutation (R464C) within the highly conserved DLAARN motif of ZAP-70 that is essential for PTK enzymatic function (Wiest et al., 1997). Analysis of TCR signaling in ST thymocytes demonstrates that R464C abrogates ZAP-70 kinase activity, but the mutant protein is expressed and can be phosphorylated. These results confirm that ZAP-70 function is required for TCR activation and development of mature murine T lymphocytes.

The absence of $CD4^+$ T cells in ZAP-70–deficient mice contrasts with their presence in humans with ZAP-70 deficiency. Although the nature of the underlying mutations in human ZAP-70 deficiency may allow for some residual PTK activity in vivo, the disparate effects of identical ZAP-70 mutations suggest instead that human and murine thymocytes have different dependencies on ZAP-70 function during development. One explanation for this discrepancy is the ability of Syk to contribute to pre-TCR and TCR signaling is substantially different between humans and mice (Au-Yeung et al., 2009). In mice, Syk expression is downregulated to levels seen in peripheral T lymphocytes following pre-TCR signaling, and the residual Syk activity in DP thymocytes of ZAP-70–deficient mice is not sufficient to rescue CD4 selection in the absence of ZAP-70 (Au-Yeung et al., 2009; Chu et al., 1998, 1999). In contrast, Syk expression has been shown to be significantly higher in human thymocytes and is not downregulated to levels seen in peripheral T lymphocytes until after positive selection has commenced (Au-Yeung et al., 2009; Chu et al., 1999; Palacios and Weiss, 2007). This explanation is supported by evidence that peripheral $CD4^+$ and $CD8^+$ T-cell development in ZAP-70–deficient mice can be restored with human Syk transgene expression in the thymus (Gong et al., 1997). As a consequence of its relative abundance in human DP thymocytes, Syk may partially compensate for loss of ZAP-70 during positive selection in ZAP-70–deficient patients. However, downregulation of Syk expression in the periphery could explain the signaling defect in ZAP-70–deficient peripheral $CD4^+$ T cells (Au-Yeung et al., 2009; Chu et al., 1999).

LCK DEFICIENCY

In contrast to ZAP-70, in which SCID patients lack peripheral $CD8^+$ T cells, human Lck deficiency has been associated with a selective CD4 lymphopenia (Goldman et al., 1998; Hubert et al., 2000; Sawabe et al., 2001). The underlying genetic defects resulting in decreased expression of functional Lck protein in a small number of sporadic cases are unknown. The clinical presentation of patients with selective CD4 lymphopenia and Lck deficiency is variable and may include SCID or common variable immunodeficiency (Goldman et al., 1998; Hubert et al., 2000; Sawabe et al., 2001).

CLINICAL AND LABORATORY FINDINGS IN LCK DEFICIENCY

In addition to CD4 lymphopenia (range 4 to 22 percent; 100 to 350 cells/mm^3), affected individuals have decreased numbers of peripheral blood lymphocytes (780–1,890 cells/mm^3) and $CD3^+$ T cells (507 to 1,200 cells/mm^3); $CD8^+$ cell percentages and numbers are normal or increased (27 to 65 percent, or 430 to 1,058 cells/mm^3). However, $CD8^+$ T cells from a Lck-deficient SCID patient poorly expressed the costimulatory molecule CD28 (Goldman et al., 1998). Although less responsive than control lymphocytes, Lck-deficient T cells do proliferate to mitogens, anti-CD3 MAb, and IL-2 and demonstrate normal alloantigen responses (Goldman et al., 1998; Hubert et al., 2000; Sawabe et al., 2001). B-cell and NK-cell numbers are appropriate for age, but some patients have hypogammaglobulinemia and require intravenous immunoglobulin therapy.

Clinically, the single Lck-deficient child with SCID had infections similar to those of other children with combined primary immunodeficiencies and required a BMT from a matched unrelated donor (Goldman et al., 1998). One elderly adult with CD4 lymphopenia and decreased Lck function presented initially with cryptococcal meningitis (Hubert et al., 2000).

LCK MUTATION ANALYSIS

The single child reported with Lck-deficient SCID was found to express an aberrantly spliced Lck mRNA lacking exon 7, which encodes the PTK ATP binding site and kinase domain (Straus and Weiss, 1992). Expression of a similar Lck mRNA lacking exon 7 is also found in the Jurkat cell line JCaM. 1 (Straus and Weiss, 1992). As a result, the JCaM. 1 cell line does not express Lck protein and exhibits profoundly defective proximal TCR signal transduction. However, decreased but detectable amount of Lck protein was seen in patient T cells by immunoblot; this signal was attributed to expression of the unstable mRNA lacking exon 7. Although less than in control lymphocytes, a significant amount of normal Lck mRNA was found in patient T cells as well. A relatively normal pattern of tyrosine phosphorylation was induced by TCR engagement despite defective expression of Lck; this is in obvious contrast to the JCaM. 1 line (Straus and Weiss, 1992). Although TCR stimulation resulted in MAPK activation and $[Ca^{2+}]_i$ mobilization at levels comparable to that of control cells, significant upregulation of CD69 expression was not observed in the patient's $CD8^+$ T cells. The underlying genetic defect resulting in defective Lck expression has not been identified.

CONCLUSIONS AND FUTURE DIRECTIONS

The defective TCR signaling observed in ZAP-70–deficient patients has established a critical role for a functional ZAP-70 PTK in normal TCR-mediated signal transduction. The failure of the TCR to mediate signals in thymocytes from mice deficient in ZAP-70 further supports the requisite role of ZAP-70 in TCR signal transduction. Despite findings that activation of Lck alone via co–cross-linking of CD4 or CD8 does not fully induce the PTK cascade and the other signaling reactions that occur as a result of TCR stimulation, a critical role for Lck in TCR signaling has been established on the basis of findings in mutant cell lines, Lck-deficient mice, and a patient. These observations indicate that both Src and Syk family PTKs play unique and critical roles in TCR signal transduction.

In summary, TCR-associated PTKs are pivotally involved in distinct developmental events required for T-cell maturation. Lck is required for signaling in thymocytes and disruption of Lck severely affects peripheral T-cell development and function in mice and humans. ZAP-70 deficiency provided the first evidence that PTKs, and ZAP-70 in particular, are required for development of normal human T-cell numbers and function. Further elucidation of the role of ZAP-70 in TCR signal transduction should be invaluable to the understanding of CD4 and CD8 selection in the thymus, as well as the pathogenesis of this form of SCID. Whether mutations in ZAP-70 result in significant autoimmunity in patients and whether other molecules involved in TCR signaling produce abnormal T-cell phenotypes in humans remain to be determined. Possible use of gene therapy as an alternative to HSCT for cure of ZAP-70 deficiency remains to be explored.

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16.

MOLECULAR BASIS OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II DEFICIENCY

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Major histocompatibility complex class II (MHCII) molecules, also called class II human leukocyte antigens (HLA) in humans, are heterodimeric transmembrane glycoproteins consisting of α and β chains. The three different human MHCII isotypes (HLA-DR, HLA-DQ, and HLA-DP) are encoded by distinct α and β chain genes clustered in the D region of the MHC on the short arm of chromosome 6. MHCII molecules occupy a central position in the adaptive immune system because they present peptides to the antigen receptor of $CD4^+$ T cells. In the thymus, MHCII–peptide complexes drive the positive and negative selection processes that guide the development of $CD4^+$ T cells. In the periphery, MHCII molecules present peptides to $CD4^+$ T lymphocytes, thereby inducing antigen-specific immune responses against pathogens and tumors. The presentation of self-peptides by MHCII molecules is also critical for the maintenance of peripheral T-cell tolerance. Given these essential functions, it is not surprising that defects in MHCII expression have profound immunopathological consequences.

Patients suffering from a rare primary immunodeficiency syndrome caused by the absence of MHCII expression were first identified in the late 1970s and early 1980s (Griscelli et al., 1980; Hadam et al., 1984, 1986; Kuis et al., 1981; Lisowska-Grospierre et al., 1983; Schuurmann et al., 1979; Touraine, 1981; Touraine et al., 1978). The lack of MHCII expression results in severely impaired cellular and humoral immune responses to foreign antigens and is consequently characterized by an extreme susceptibility to viral, bacterial, fungal, and protozoal infections, primarily of the respiratory and gastrointestinal tracts (reviewed in Elhasid and Etzioni, 1996; Griscelli et al., 1993; Klein et al., 1993). Severe malabsorption with failure to thrive ensues, often leading to death in early childhood.

The disease was formally named *major histocompatibility complex class II deficiency* (MHCII deficiency) (Rosen et al., 1992) and has been assigned the MIM (Mendelian Inheritance in Man) number 209920. It is also frequently referred to as the bare lymphocyte syndrome (BLS). However, the term *BLS* was first used to describe a defect in MHC class I (MHCI) expression in patients in which MHCII expression was not examined (Touraine et al., 1978), and it has been used synonymously for all defects involving expression of MHCI (BLS type I), MHCII (BLS type II), or both (BLS type III) (Touraine et al., 1992). Here we will discuss only the syndrome associated with a defect in MHCII expression, and will thus use the term *MHCII deficiency*.

More than 100 unrelated patients have been reported worldwide. The majority of patients are of North African origin (Algeria, Tunisia, and Morocco) (Griscelli et al., 1993; Klein et al., 1993; Lisowska-Grospierre et al., 1994). The remaining patients are of diverse ethnic backgrounds, including Spain, Italy, Turkey, France, Holland, the United States, Israel, Saudi Arabia, and Pakistan (Bejaoui et al., 1998; Casper et al., 1990; Clement et al., 1988; Haas and Stiehm, 1987; Hadam et al., 1984, 1986; Hume et al., 1989; Kuis et al., 1981; Lisowska-Grospierre et al., 1994; Peijnenburg et al., 1995; Wiszniewski et al., 2000). There is a high incidence of consanguinity in the affected families (Lisowska-Grospierre et al., 1994).

MHCII deficiency has an autosomal recessive mode of inheritance. A comparison between the patterns of inheritance of the disease and the MHC genotype in affected families has demonstrated that the genetic lesions responsible for MHCII deficiency lie outside the MHC (de Preval et al., 1985; Griscelli et al., 1993). This suggested that the disease is due to defects in transacting regulatory factors required for expression of MHCII genes. Elucidation of the molecular defects responsible for MHCII deficiency has confirmed this. The affected genes are now indeed known to encode

regulatory factors controlling transcription of MHCII genes. Four regulatory genes—*CIITA* (MIM number 600005), *RFXANK* (MIM number 603200), *RFX5* (MIM number 601863), and *RFXAP* (MIM number 601861)—have been isolated and shown to be mutated in MHCII deficiency patients (reviewed in Masternak et al., 2000a; Reith and Mach, 2001).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Comprehensive accounts of the clinical and pathological manifestations associated with MHCII deficiency have been reviewed previously (Elhasid and Etzioni, 1996; Griscelli et al., 1993; Klein et al., 1993). The major findings are summarized in Table 16.1. Clinical manifestations include primarily septicemia and recurrent infections of the gastrointestinal, pulmonary, upper respiratory, and urinary tracts. The patients are prone to bacterial, fungal, viral, and protozoal infections. The fungus isolated most frequently from the lung is *Pneumocystis jirovoci*, and the most common protozoal agent is *Cryptosporidium parvum*. Infections start within the first year of life; the mean age at first infection is 4.3 months. Subsequent evolution of the disease is characterized by an inexorable progression of the infectious complications until death ensues. Although some children reach puberty and a few attain adulthood, the majority die before the age of 10 years (see Table 16.4). All of the pathological manifestations of MHCII deficiency are related to the infectious complications, and with the exception of the absence of MHCII expression, there are no features that are specific for this particular immunodeficiency.

Table 16.1 CLINICAL MANIFESTATIONS

	PATIENTS	
MANIFESTATION	NUMBER	%
Repeated severe infections	81	99
Protracted diarrhea	68	83
Lower respiratory tract infections	66	80
Failure to thrive	56	68
Upper respiratory tract infections	55	67
Severe viral infections	49	58
Mucocutaneous candidiasis	24	29
Progressive liver disease	13	16
Cryptosporidiosis	13	16
Sclerosing cholangitis	7	9
Autoimmune cytopenia	5	6

Mean age at first infection is 4.3 months (range, 6 weeks to 12 months). Included are 63 patients from Hospital Necker-Enfants Malades (Picard, unpublished data), 9 Tunisian patients (Bejaoui et al., 1998), and 10 patients reported by Saleem et al. (2000). Not included are four patients with an atypical clinical presentation in two unrelated families belonging to complementation group A (Quan et al., 1999; Wiszniewski et al., 2001).

Bacterial infections in various locations are dominant. These include intestinal infections, pneumonitis, bronchitis, and septicemia. The bacteria isolated most frequently from stools include *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Campylobacter jejuni*, *Proteus mirabilis*, and *P. morgani*. In almost all patients, bacterial infections of the intestinal tract, together with *Candida albicans*, *Giardia lamblia*, and *C. parvum* infections, are responsible for protracted diarrhea, malabsorption, and failure to thrive as of the first year of life. As in CD40L deficiency, intestinal and hepatic involvement due to *C. parvum* colonization appears to be more frequent in MHCII deficiency than it is in other immunodeficiencies. Histological examinations of the intestinal mucosa revealed varying degrees of villous atrophy, frequently associated with intraepithelial infiltration by lymphocytes, macrophages, and plasma cells.

Hepatic involvement is frequent, but the manifestations are not uniform. Many patients exhibit either symptoms suggestive of viral hepatitis, or hepatic involvement resulting from cholangitis, parenteral nutrition, or the use of hepatotoxic drugs. The most frequent cause of hepatitis is infection with cytomegalovirus (CMV). Pseudosclerosing cholangitis is a frequent complication of chronic *C. parvum* infection in patients older than 4 years of age.

Recurrent bronchopulmonary infections have been observed in all patients. Diffuse interstitial pneumonia caused by *P. jirovici* or viruses (CMV, respiratory syncytial virus, enterovirus, adenovirus) are frequently observed. The most frequently isolated bacteria during pneumonia and/or sepsis were *Streptococcus pneumoniae, Staphylococcus aureus*, and *Haemophilus influenzae*. Recurrent upper respiratory tract infections including otitis, rhinitis, and sinusitis were observed in almost all patients.

Neurological manifestations due to viral infections have been diagnosed in a number of patients. These include poliomyelitis, meningoencephalitis, and chronic lymphocytic meningitis caused by live attenuated poliovirus vaccination, herpes simplex virus, enterovirus, and adenovirus.

Hematological manifestations are characterized by transient eosinophilia, neutropenia, autoimmune hemolytic anemia, and thrombopenia.

The clinical features vary considerably from one patient to another. This variability does not show any correlation with the genetic heterogeneity in the cause of the disease (see below). Interestingly, several patients having an atypical clinical course have been identified (Hauber et al., 1995; Nekrep et al., 2002; Prod'homme et al., 2003; Wiszniewski et al., 2001; Wolf et al., 1995). The clinical manifestations in these patients are less severe or even absent, and their survival is considerably longer. Several patients over 20 years of age have been identified (see Table 16.4). The unusual capacity of these patients to cope with infections depends on genetic, immunological, and/or environmental differences that remain largely unknown. However, in certain cases it has been attributed to partial loss-of-function mutations in the affected genes (Nekrep et al., 2002; Prod'homme et al., 2003; Wiszniewski et al., 2001).

LABORATORY FINDINGS AND IMMUNOLOGICAL FEATURES

The immunological characteristics of MHCII deficiency (reviewed in Griscelli et al., 1993; Klein et al., 1993) are summarized in Table 16.2. Most of the immunological manifestations can be accounted for by the absence of antigen presentation via MHCII molecules. The most striking and constant consequence of the defect in MHCII expression is, as expected, the absence of cellular and humoral immune responses to foreign antigens. Patients are unable to mount T-cell–mediated immune responses to specific antigens, as assessed by delayed-type hypersensitivity skin tests. This correlates with an absence of T-cell responses in vitro to antigens with which the patients had been immunized or sensitized to by infections. Also consistent with the absence of MHCII expression is the finding that lymphocytes from patients have a decreased capacity to stimulate HLA-nonidentical lymphocytes in the mixed lymphocyte reaction.

Patients have T-cell lymphopenia. $CD4^+$ T-cell counts are reduced, whereas $CD8^+$ T-cell counts can be normal or decreased. The diminished $CD4^+$ T-cell count reflects abnormal development of $CD4^+$ thymocytes, resulting from defective MHCII expression in the thymus. Surprisingly, however, the remaining $CD4^+$ T-cell population appears to be phenotypically and functionally normal. The patient's $CD4^+$ T cells exhibit only relatively minor differences with respect to the normal TCR repertoire (Henwood et al., 1996; Rieux Laucat et al., 1993) and behave normally in terms of alloreactivity and proliferative responses to mitogens (Griscelli et al., 1993; Klein et al., 1993).

Although numbers of circulating B lymphocytes are normal, humoral immunity is severely impaired. Most patients have hypogammaglobulinemia, some have a decrease in one or two immunoglobulin isotypes, and certain exhibit a hyper-IgM profile. Antibody responses to immunizations and infections by microbial agents are absent, except for the production of T-independent antibodies against encapsulated bacteria. Interestingly, autoantibodies associated with autoimmune disorders have been found in several patients. In conclusion, most patients were severely immunodeficient, with low $CD4^+$ T-cell counts and profoundly impaired antigen-specific T- and B-cell responses.

Table 16.2 SUMMARY OF IMMUNOLOGICAL FINDINGS

PARAMETER MEASURED	FINDINGS	PATIENTS
HLA Class II Expression[†]		n = 63
B cells	<1%	76%
B cells	1–43%*	24%
Monocytes	< 1%	89%
Monocytes	1–17%*	11%
HLA Class I Expression[†]		n = 38
Mononuclear cells	Reduced	79%
T-cell Immunity		n = 59
$CD4^+$ lymphopenia		91.5%
Lymphocyte proliferative responses to:		
- PHA	Normal	97%
- Antigen	Absent or strongly reduced	100%
B-cell Immunity		n = 34
Serum immunoglobulins		
- IgG	Decreased	82%
- IgM	Decreased	65%
- IgM	Increased	9%
- IgA	Decreased	50%
Antibody Response		
DTP immunizations	Negative	95%

Data are from 63 patients treated at Hospital Necker-Enfants Malades between 1977 and 2009 (Picard, unpublished data).

[†]Tested by fluorescence-activated cell sorting or microscopy.

*Numbers indicate the percentage of "dull" stained cells.

MOLECULAR BASIS

DEFECTIVE EXPRESSION OF MHCII MOLECULES

The hallmark of MHCII deficiency is the absence of MHCII molecules on the surface of all cells that normally express them, and the demonstration of this lack of expression remains the mainstay of diagnosis. In normal individuals, two modes of MHCII expression, constitutive and inducible, are observed (reviewed in Glimcher and Kara, 1992; Guardiola and Maffei, 1993; Mach et al., 1996). Constitutive expression is largely restricted to professional antigen-presenting cells (B lymphocytes, cells of the monocyte/macrophage lineage, and dendritic cells) and epithelial cells in the thymus. Inducible expression can be observed in virtually all cell types in response to a variety of stimuli, of which the most potent is interferon-γ (IFN-γ). In MHCII-deficient patients, both the constitutive and inducible expression modes are abolished (reviewed in Griscelli et al., 1993; Klein et al., 1993, Mach et al., 1996). The absence of MHCII molecules concerns all professional antigen-presenting cells, including B lymphocytes, cells of the macrophage/monocyte lineage, and dendritic cells. Activated T cells remain MHCII negative. Thymic epithelial cells, and endothelial and epithelial cells of the intestinal and bronchial mucosa, also lack MHCII molecules. Finally, MHCII expression cannot be restored in any cell type by stimulation with IFN-γ.

The inability to express MHCII molecules constitutively or in response to IFN-γ has been demonstrated in vitro using peripheral blood lymphocytes (PBLs), Epstein-Barr virus (EBV)-transformed B-cell lines, interleukin-2 (IL-2)-dependent T-cell lines, and primary or transformed fibroblasts derived from MHCII deficiency patients (de Preval et al., 1985, 1988; Griscelli et al., 1993; Klein et al., 1993; Lisowska-Grospierre et al., 1985). Studies on

EBV-transformed B-cell lines from patients indicated that expression of MHCII genes could not be detected at the levels of the cell surface, intracellular protein, or mRNA (de Preval et al., 1985; Lisowska-Grospierre et al., 1985). In fibroblasts, the expression of MHCII genes cannot be induced by IFN-γ (de Preval et al., 1988). The lack of constitutive and inducible expression concerns all classical MHCII genes, including those encoding the α and β chains of HLA-DR, HLA-DP, and HLA-DQ (de Preval et al., 1985, 1988; Lisowska-Grospierre et al., 1985). There are also defects in expression of the invariant chain (*Ii*) gene (*CD74*), and the *HLA-DM* and *HLA-DO* genes (Kern et al., 1995; Nagarajan et al., 2002; Nocera et al., 1993; Seguín-Estévez et al., 2009; Taxman et al., 2000), which encode proteins required for antigen presentation by MHCII molecules (Alfonso and Karlsson, 2000; Busch et al., 2005; Cresswell, 1996; Denzin et al., 2005).

Although a complete absence of MHCII molecules is the general rule, certain patients have a "leaky" phenotype (Griscelli et al., 1993; Klein et al., 1993). Residual levels of MHCII expression have been observed in several patients on various cell types, including B cells, monocytes, activated T cells, Langerhans cells, intestinal endothelial cells, and thymic stromal and dendritic cells (Hadam et al., 1986; Klein et al., 1993; Schuurman et al., 1985). Low levels of expression of certain MHCII genes have also been observed in EBV-transformed B-cell lines and IFN-γ-treated fibroblasts derived from certain patients (Griscelli et al., 1993; Klein et al., 1993; Lennon-Dumenil et al., 2001; Peijnenburg et al., 1995).

In addition to the profound defect in MHCII expression, a reduction in cell-surface expression of MHCI and β2 microglobulin (β2m) is observed on fresh PBLs, cultured fibroblasts, SV40-transformed fibroblasts, and EBV-transformed B-cell lines from MHCII deficiency patients (Griscelli et al., 1980; Hadam et al., 1984, 1986; Sabatier et al., 1996; Schuurmann et al., 1979; Touraine et al., 1978). A reduction in MHCI expression is observed to a variable degree in over 75% of the patients (Table 16.2) (Klein et al., 1993).

MHCII DEFICIENCY IS DUE TO REGULATORY DEFECTS IN MHCII GENE TRANSCRIPTION

Direct measurements of the transcriptional activity of MHCII genes have demonstrated that the lack of expression in patients is due to a deficiency in transcription (Reith et al., 1988). This finding suggested that the disease is due to defects in regulatory factors controlling transcriptional activation of MHCII genes. Direct evidence for this interpretation was provided by four independent observations. First, family studies demonstrated that the genetic lesions responsible for the disease do not cosegregate with the MHC (de Preval et al., 1985; Griscelli et al., 1993). Second, reporter genes fused to the DNA sequences controlling transcription of MHCII genes remain silent upon transfection into cells from patients (Hasegawa et al., 1993; Riley and Boss, 1993). Third, nuclear extracts from patients' cells are unable to activate transcription from MHCII promoters in in vitro transcription experiments (Durand et al., 1994). Finally, expression of the endogenous MHCII genes of the patients' cells can be reactivated in somatic cell fusion experiments (see below).

GENETIC HETEROGENEITY IN MHCII DEFICIENCY

Somatic cell-fusion experiments performed with cell lines derived from patients first indicated that the causes of MHCII deficiency are genetically heterogeneous (Bénichou and Strominger, 1991; Hume and Lee, 1989; Lisowska-Grospierre et al., 1994; Seidl et al., 1992). Several experimentally generated cell lines (Accolla, 1983; Calman and Peterlin, 1987; Gladstone and Pious, 1978) exhibiting an MHCII-negative phenotype indistinguishable from the patients' cell lines were also included in the cell-fusion studies. The results of these experiments indicated that the patients and experimentally derived cell lines can be assigned to four complementation groups (Table 16.3, groups A to D), reflecting the existence of mutations in four MHCII regulatory genes. Most patients (43 unrelated families) fall into complementation group B (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a, Picard, unpublished data). Group A contains patients from ten different families (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a) as well as three experimentally derived mutants, of which the best known is RJ2.2.5 (Accolla, 1983). Group C contains patients from seven families (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a). Group D was first defined by cell-fusion experiments with the experimentally generated cell line 6.1.6 (Gladstone and Pious, 1978). Subsequent cell-fusion experiments and isolation of the gene affected in 6.1.6 permitted the classification of patients from seven unrelated families in this group (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a).

The spectrum of clinical and immunological manifestations associated with the disease encompasses all four complementation groups (Griscelli et al., 1993; Klein et al., 1993). No distinctive features restricted to only certain complementation groups have been identified. Despite the genetic heterogeneity in the cause of MHCII deficiency, the syndrome is therefore phenotypically homogeneous.

ATYPICAL FORM OF MHCII DEFICIENCY

Twin brothers exhibiting an atypical and less severe form of MHCII deficiency have been described (Douhan et al., 1996; Hauber et al., 1995; Wolf et al., 1995). In contrast to the situation observed in the "classical" form of MHCII deficiency, defective expression in these patients does not concern all MHCII genes equally and does not affect all cell types to the same extent. In EBV-transformed B cells from these patients, the *HLA-DRB*, *HLA-DQB*, and *HLA-DPA* genes are silent whereas the *HLA-DRA*, *HLA-DQA*, and *HLA-DPB* genes are expressed. In mononuclear cells, there is a significant level of *HLA-DRB*, *HLA-DQB*, and *HLA-DPA* expression. Investigations of MHCII-dependent immune functions in these patients indicated the presence of competent MHCII-positive antigen-presenting cells (Hauber et al., 1995; Wolf et al., 1995). Impairment of the immune response is

Table 16.3 BIOCHEMICAL AND MOLECULAR DEFECTS IN MHCII-DEFICIENCY PATIENTS AND REGULATORY MUTANTS FROM COMPLEMENTATION GROUPS A TO D

		MHCII DEFICIENCY COMPLEMENTATION GROUPS*			
	WILD TYPE	A (II)	B (I)	C (IV)	*D (III)*
Prototypical Patient		**BLS-2**	**BLS-1, Ra**	**SJO**	**DA, ABI, ZM**
Number Of Unrelated Families†		**10**	**43**	**7**	**7**
Prototypical In Vitro Mutant		**RJ2.2.5**	**None**	**G1B**	**6.1.6**
Number Of In Vitro Mutants†		**3**	**NONE**	**1**	**1**
MHCII Expression	+	−	−	−	−
MHCII Promoter Activity‡	+	−	−	−	−
Binding Of RFX‡	+	+	−	−	−
Dnasei Hypersensitive Sites‡	+	+	−	−	−
Promoter Occupancy In Vivo‡	+	+	−	−	−
Affected Gene		*CIITA*	*RFXANK***	*RFX5*	*RFXAP*
Mim Number		**600005**	**603200**	**601863**	**601861**
Mrna Sequence Entry		**X74301**	**AF094760**	**X85786**	**Y12812**
Chromosomal Localization		**16P13**	**19P12**	**1Q21.1−21.3**	**13Q14**
Protein		**CIITA**	**RFXANK****	**RFX5**	**RFXAP**
size of protein (amino acids)		**1130**	**269**	**616**	**272**

*A–D, nomenclature for complementation groups used in Bénichou and Strominger (1991) and Lisowska-Grospierre et al. (1994); I–IV, alternative nomenclature used in Seidl et a bl. (1992).

†Numbers are based on somatic cell-fusion experiments and/or mutation analysis of the affected genes.

‡Tested on at least one cell line in each complementation group.

**Also called RFX-B in Nagarajan et al. (1999).

consequently much less evident than in classical MHCII deficiency. Characterization of the genetic defect in these patients has demonstrated that they belong to complementation group C (Nekrep et al., 2002) rather than to a fifth complementation group as initially believed (Douhan et al., 1996).

BIOCHEMICAL HETEROGENEITY IN MHCII DEFICIENCY

MHCII expression is regulated primarily at the level of transcription by a short DNA segment situated immediately upstream of the transcription initiation site of each MHCII gene (reviewed in Benoist and Mathis, 1990; Mach et al., 1996). This promoter proximal region contains four cis-acting DNA elements—called the *S*, *X*, *X2*, and *Y* boxes—that are highly conserved in their sequence and organization with respect to orientation, order, and spacing relative to each other (Fig. 16.1A). This conservation is apparent not only in all MHCII genes from all species that have been examined, but also in the invariant chain, *DM* and *DO* genes.

In the hope of elucidating the molecular mechanisms controlling transcription of MHCII genes and identifying the regulatory genes that are mutated in MHCII deficiency, numerous studies were performed to identify and isolate DNA binding proteins that interact specifically with MHCII promoters (reviewed in Mach et al., 1996). These studies led to the identification of several key transcription factors, particularly the X box binding complex called RFX (regulatory factor X) (Reith et al., 1988).

RFX was first identified in nuclear extracts from MHCII-positive B-cell lines on the basis of its ability to bind in vitro to the X box of MHCII promoters (Reith et al., 1988). It was purified by affinity chromatography and shown to be a heteromeric protein consisting of three subunits of 75kDa, 36kDa, and 33kDa (Durand et al., 1994, 1997; Masternak et al., 1998). The DNA-binding activity of RFX was analyzed extensively by in vitro studies using nuclear extracts from cell lines derived from MHCII deficiency patients and from experimentally generated regulatory mutants (Durand et al., 1994; Hasegawa et al., 1993; Herrero-Sanchez et al., 1992; Reith et al., 1988; Stimac et al., 1991). Based on the presence or absence of RFX binding activity, two types of mutant cells were recognized (Table 16.3, Fig. 16.1); RFX binding activity was normal in cell lines classified in complementation group A but deficient in cell lines assigned to complementation groups B, C, and D (reviewed in Mach et al., 1996).

Examination of DNAse I hypersensitive sites (Gonczy et al., 1989) and in vivo footprint experiments (Kara and Glimcher, 1991, 1993) revealed that the lack of RFX binding activity correlates with a bare promoter in vivo (Table 16.3, Fig. 16.1) (reviewed in Mach et al., 1996). In RFX-positive cells (normal MHCII-positive B cells as well as B cells from complementation group A), MHCII promoters exhibit characteristic DNAse I hypersensitive sites and are occupied by DNA-binding proteins in vivo. In RFX-deficient B cells (complementation groups

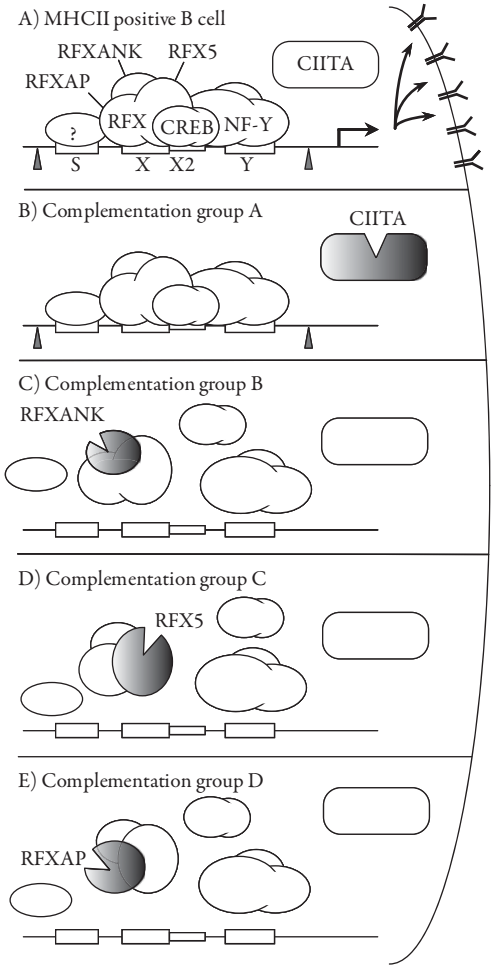

Figure 16.1 Molecular defects in MHCII deficiency. MHCII promoter occupancy and transcription status are represented schematically for normal B cells (A) and B cells from complementation groups A to D (B–E). Open boxes represent the S, X, X2, and Y sequences. RFX, CREB, and NF-Y bind to the X, X2, and Y boxes to form a nucleoprotein complex called the MHCII enhanceosome. The identity of the protein that binds to the S box remains unknown. The MHCII enhanceosome forms a platform to which CIITA is recruited (see Fig. 16.2). Arrowheads represent DNAse I hypersensitive sites that flank the S-X-X2-Y region and are diagnostic of promoter occupation. CIITA is mutated in complementation group A. Mutations in CIITA do not modify promoter occupation in vivo. In complementation groups B, C, and D, the RFX5, RFXANK, and RFXAP subunits of RFX are mutated. A deficiency in RFX leads to a bare promoter in vivo.

B-D), the DNAse I hypersensitive sites are missing and all of the MHCII promoter elements are unoccupied.

IDENTIFICATION OF *CIITA*, THE GENE AFFECTED IN COMPLEMENTATION GROUP A

A genetic approach relying on cDNA expression cloning was developed to identify the genes affected in MHCII deficiency (Steimle et al., 1993). Briefly, cell lines established from patients, or experimentally generated cell lines, were transfected with cDNA expression libraries and complemented cells carrying cDNA clones capable of restoring MHCII expression were selected for. This approach was first successful in the case of RJ2.2.5, an experimentally generated cell line from complementation group A. Complementation of RJ2.2.5 led to isolation of the MHCII transactivator CIITA (Steimle et al., 1993). The human *CIITA* gene is localized on chromosome 16 (16p13), and the corresponding mouse gene is situated in a syntenic region of mouse chromosome 16. This localization is in accordance with the findings that regulatory genes present on human and mouse chromosomes 16 are required for expression of MHCII genes (Accolla et al., 1986; Bono et al., 1991).

IDENTIFICATION OF *RFXANK,* THE GENE AFFECTED IN COMPLEMENTATION GROUP B

The gene affected in complementation group B, which contains the largest number of patients (Table 16.3), was isolated by a biochemical approach based on purification of the RFX complex (Masternak et al., 1998). It was baptized *RFXANK* because the protein that it encodes contains four ankyrin repeats, which are well-known protein–protein interaction motifs. The same gene was subsequently also called *RFX-B* (Nagarajan et al., 1999). *RFXANK* encodes the 33kDa subunit of the RFX complex (Fig. 16.1). The chromosomal localization of *RFXANK is* 19p12.

IDENTIFICATION OF *RFX5,* THE GENE AFFECTED IN COMPLEMENTATION GROUP C

The same complementation approach used to isolate CIITA was successful in elucidating the molecular defect leading to the lack of RFX binding activity in patients from complementation group C. Complementation of a cell line derived from patient SJO (Casper et al., 1990) led to the isolation of a cDNA encoding the 75kDa subunit of RFX (Fig. 16.1) (Steimle et al., 1995). The gene was called *RFX5* because it encoded the fifth member of a family of X box binding proteins sharing a highly characteristic DNA binding domain (DBD) (Aftab et al., 2008; Emery et al., 1996; Steimle et al., 1995). This RFX DBD has been strongly conserved in evolution and has been identified in a variety of proteins having diverse regulatory functions in organisms ranging from yeast to humans (Aftab et al., 2008; Emery et al., 1996). The structure of the DBD of one member of the RFX family (RFX1) has been determined and shown to belong to the winged-helix subfamily of helix-turn-helix (HTH) proteins (Gajiwala et al., 2000). Surprisingly, the RFX1 DBD binds DNA in a fashion that is radically different from that observed for all other known HTH proteins (Gajiwala et al., 2000). The amino acids implicated in site-specific binding of the RFX1 DBD are strongly conserved in RFX5, implying that the latter interacts with its X box target site in a similar fashion (Gajiwala et al., 2000). The human *RFX5* gene is situated in a subcentromeric region of the long arm of chromosome 1 (Villard et al., 1997b). The corresponding mouse gene maps to a syntenic region of chromosome 3.

IDENTIFICATION OF *RFXAP*, THE GENE AFFECTED IN COMPLEMENTATION GROUP D

The experimentally generated cell line 6.1.6 (Gladstone and Pious, 1978) was initially the only representative of complementation group D (Bénichou and Strominger, 1991; Hume and Lee, 1989; Lisowska-Grospierre et al., 1994; Seidl et al., 1992). The gene affected in 6.1.6 was isolated by a biochemical approach based on purification of the RFX complex. It was shown to encode a novel protein corresponding to the 36 kDa subunit of RFX (Fig. 16.1) (Durand et al., 1997). This protein was called RFX-associated protein (RFXAP) because it is a subunit of the RFX complex but does not contain the DBD characteristic of the RFX family of DNA binding proteins (Durand et al., 1997). Isolation of the *RFXAP* gene permitted the identification of MHCII-deficiency patients belonging to complementation group D (Durand et al., 1997; Villard et al., 1997a). The *RFXAP* gene is localized on the long arm of chromosome 13 (Villard et al., 1997a).

ELUCIDATION OF THE MOLECULAR DEFECT IN ATYPICAL PATIENTS

The gene affected in the atypical patients turned out to be RFX5, thereby placing these patients in complementation group C (Nekrep et al., 2002). The defect in the patients is a point mutation that replaces a critical residue in the DBD of RFX5. The unusual pattern of residual MHCII expression and atypical phenotype of these patients are probably a result of the fact that this mutation does not compromise binding of RFX to all MHCII promoters to the same extent (Nekrep et al., 2002).

FUNCTION OF THE AFFECTED MHCII REGULATORY FACTORS

STRUCTURE AND MODE OF ACTION OF CIITA

CIITA contains several well-defined protein motifs (see Fig. 16.3) (reviewed in Harton and Ting, 2000; Reith and Mach, 2001; Ting and Trowsdale, 2002). Its N-terminus contains a region rich in acidic amino acids, followed by three segments rich in proline, serine, and threonine. The central part of the protein contains a nucleotide binding and oligomerization domain (NOD), which comprises a GTP binding domain (GBD). Finally, the C-terminus contains a leucine-rich repeat (LRR)-based protein–protein interaction motif. These features are all required for the function of CIITA, although their precise roles have been determined only partially (see below).

CIITA is a member of a large family of proteins characterized by the presence of a centrally placed nucleotide binding domain (NBD) and a C-terminal LRR domain (Harton et al., 2002; Inohara et al., 2005; Meylan et al., 2006; Ting et al., 2008). Although a variety of different names have been used to describe this family, a recent initiative has proposed the unifying designation NLR (for NBD and LRR containing) (Ting et al., 2008). In a manner analogous to CIITA for MHCII genes, another family member, NLRC5, functions as a transactivator of MHCI genes (Kobayashi and van den Elsen, 2012) The other family members are cytoplasmic proteins playing roles in apoptosis, inflammation, and innate immunity (Inohara et al., 2005; Meylan et al., 2006; Ting et al., 2008). Several are associated with immunological and inflammatory disorders (Inohara et al., 2005; Meylan et al., 2006; Ting et al., 2008).

CIITA does not contain a recognizable DNA-binding domain and does not bind to DNA. However, chromatin immunoprecipitation experiments have demonstrated that CIITA is nevertheless recruited to the promoters of the MHCII, *Ii*, *HLA-DM*, and *HLA-DO* genes in vivo (Beresford and Boss, 2001; Hake et al., 2000; Krawczyk et al., 2008; Masternak et al., 2000b, 2003; Masternak and Reith, 2002). Recruitment of CIITA to MHCII promoters is mediated by multiple protein–protein interactions with DNA-bound factors (Fig. 16.2A). Factors bound to the S, X, X2, and Y boxes are all required for the recruitment of CIITA (DeSandro et al., 2000; Hake et al., 2000; Masternak et al., 2000b; Zhu et al., 2000).

Once tethered to MHCII promoters, CIITA is believed to activate transcription via N-terminal transcription activation domains (Fig. 16.2A) (reviewed in Harton and Ting, 2000; Reith and Mach, 2001; Ting and Trowsdale, 2002). The acidic and proline/serine/threonine-rich regions found in the N-terminus of CIITA are reminiscent of the transcription activation domains present in other transcription factors. Moreover, they can activate transcription when fused to heterologous DNA-binding proteins and can be replaced by activation domains from other transcription factors (Riley et al., 1995; Zhou and Glimcher, 1995).

Several mechanisms have been implicated in the activation of transcription by CIITA (Fig. 16.2A). First, CIITA can interact with the general transcription factors $TF_{II}B$, $TAF_{II}32$, and $TAF_{II}70$, suggesting that it activates transcription initiation by recruiting the general transcription machinery (Fontes et al., 1997; Mahanta et al., 1997; Masternak and Reith, 2002; Masternak et al., 2003). Second, it has been proposed that CIITA stimulates promoter clearance by RNA polymerase II and transcription elongation by promoting the recruitment of $TF_{II}H$ and P-TEFb (Kanazawa et al., 2000; Mahanta et al., 1997; Spilianakis et al. 2003). Third, CIITA has been proposed to enhance chromatin accessibility by inducing recruitment of the ATP-dependent chromatin remodeling factors Brg1 and Brm1 (Mudhasani and Fontes, 2002; Spilianakis et al. 2003). Fourth, CIITA is believed to promote the acetylation of histones H3 and H4 by recruiting histone acetyl transferases, such as pCAF, GCN5, CBP, and SRC1 (Beresford and Boss, 2001; Fontes et al., 1999; Kretsovali et al., 1998; Masternak and Reith, 2002; Masternak et al., 2003; Spilianakis et al., 2000; Tzortzakaki et al., 2003; Zhu and Ting, 2001). CIITA has also been reported to have an intrinsic histone acetyl transferase activity (Raval et al., 2001). Finally, it has been reported that CIITA can promote methylation of histone H3 by recruiting the histone methyl transferase CARM1 (Zika et al., 2005).

Analysis of the intracellular distribution of CIITA has shown that a substantial amount of the protein is localized in

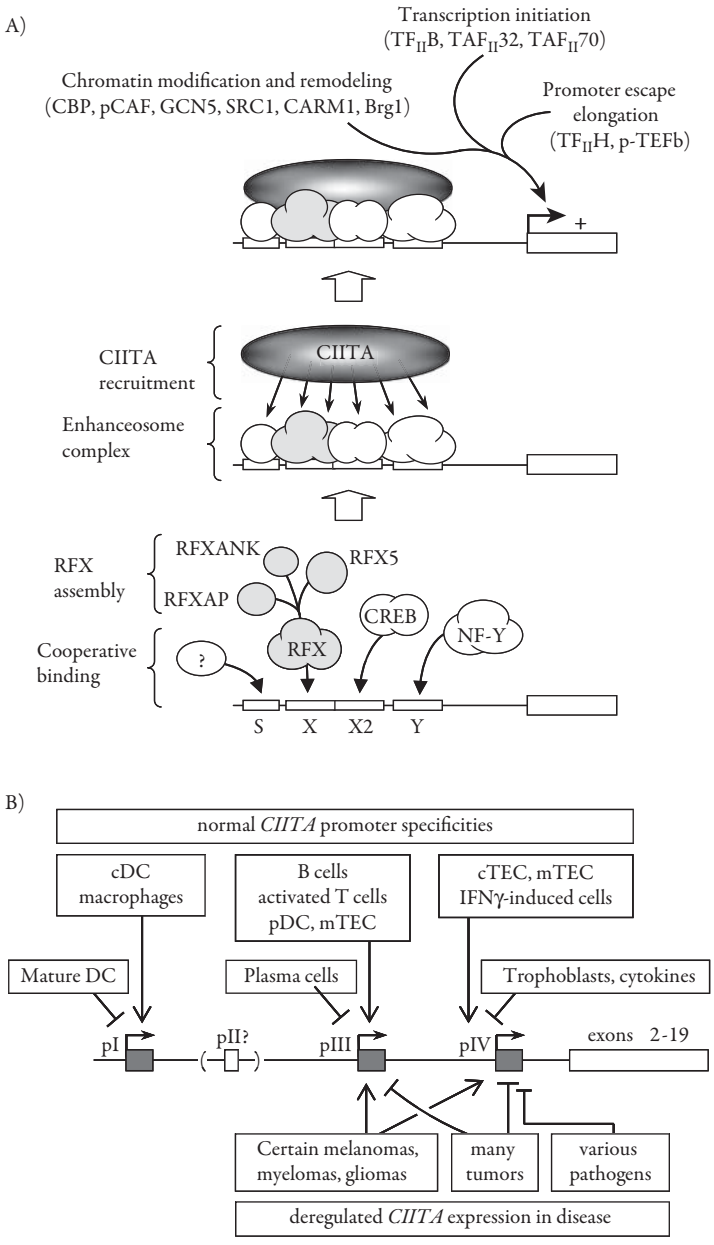

Figure 16.2 Overview of MHCII gene regulation. (A) The ubiquitously expressed factors RFX, CREB, and NF-Y assemble into an enhanceosome complex on the promoters of MHCII genes. The highly regulated non–DNA-binding coactivator CIITA is recruited to the enhanceosome by multiple protein–protein interactions. CIITA is believed to promote chromatin remodeling by interacting with histone acetyl- and methyl-transferases, and nucleosome remodeling factors. CIITA can also recruit components of the general transcription initiation machinery and factors involved in promoter clearance and transcription elongation. Brg1, Brahma-related gene 1; CARM1, coactivator-associated arginine methyltransferase 1; CBP, CREB-binding protein; CREB, cAMP response element-binding protein; GCN5, general control of amino acid synthesis 5; NF-Y, nuclear factor Y; pCAF, p300/CBP-associated factor; P-TEFb, positive transcription elongation factor b; SRC1, steroid receptor coactivator 1; TAF, TBP-associated factor; TBP, TATA-box-binding protein; $TF_{II}B$ and $TF_{II}H$, general transcription factors for RNA polymerase II. (B) Regulation of CIITA expression. Transcription of the *CIITA* gene is controlled by four independent promoters (pI, pII, pIII, and pIV) preceding four alternative first exons. Three of these promoters (pI, pIII, and pIV) are conserved in the mouse *CIIta* gene. These three promoters exhibit well-defined cell-type specificities and responsiveness to IFN-γ. pI is used mainly in macrophages and conventional dendritic cells (cDC). pIII is used in B cells, activated T cells, plasmacytoid dendritic cells (pDC), and medullar thymic epithelial cells (mTEC). pIV is used in both cortical and medullar thymic epithelial cells (cTEC, mTEC) and is induced by IFN-γ in cells of nonhematopoietic origin. pI, pIII, and pIV are silenced in mature DCs, plasma cells, and trophobasts, respectively. Activation of pI is also inhibited by certain anti-inflammatory cytokines. Aberrant activation or silencing of pIII and pIV is observed in various tumors and infectious diseases. The function of pII is not known.

the nucleus. Several regions within CIITA have been implicated in nuclear targeting of the protein. Three nuclear localization signals (NLS) have been identified (Fig. 16.3). One of these lies within a region that was found to be deleted in a BLS patient (Cressman et al., 1999). The GTP-binding domain also appears to be required for nuclear import (Harton et al., 1999). Finally, a detailed mutational analysis of the LRR region has shown that it is important for directing CIITA to the nucleus (Hake et al., 2000).

Several studies have shown that CIITA can associate with itself to form homomeric complexes. Although there is some controversy concerning the precise sequences that mediate self-association, the LRR and NOD domains have been implicated by several studies (Kretsovali et al., 2001; Linhoff et al., 2001; Sisk et al., 2001). The precise role of self-association of CIITA remains unknown.

TARGET GENE SPECIFICITY OF CIITA

Classical MHCII genes, and the *Ii* (CD74), *HLA-DM*, and *HLA-DO* genes required for antigen presentation by MHCII molecules, are the best-documented target genes of CIITA (Chang and Flavell, 1995; Kern et al., 1995; Krawczyk et al., 2008; Steimle et al., 1993). As all clinical manifestations and immunological abnormalities associated with MHCII deficiency can be explained by defective expression of these genes, it appeared unlikely that CIITA would play essential roles in other systems. It therefore came as a surprise when a series of studies proposed that CIITA can modulate the expression of numerous other genes involved in diverse functions within and outside the immune system (reviewed in LeibundGut-Landmann et al., 2004a). Collectively, these reports suggested that CIITA might have widespread functions extending beyond its role in the control of MHC

expression, a notion that was at odds with the highly specific defects observed in MHCII deficiency. To address this discrepancy, genome-wide searches were recently performed to define the complete set of CIITA target genes (Krawczyk et al., 2007, 2008). These searches identified only a handful of new bona-fide target genes, several of which encode proteins that are known or likely to be implicated in processes related to antigen presentation. CIITA was thus confirmed to be remarkably dedicated for the regulation of genes implicated in MHCII-mediated antigen presentation.

EXPRESSION OF CIITA

CIITA functions as a "master regulator" of MHCII genes. Numerous studies have demonstrated that it is mainly the expression pattern of CIITA that governs the cell-type specificity, induction, and level of MHCII expression. (reviewed in Harton and Ting, 2000; Reith and Mach 2001; Reith et al., 2005; Ting and Trowsdale, 2002). MHCII expression by antigen-presenting cells (B cells, macrophages, and dendritic cells), activated T cells, and thymic epithelial cells is driven by CIITA expression (Chang et al., 1996b; Irla et al., 2008; Landmann et al., 2001; LeibundGut-Landmann et al., 2004b; Otten et al., 1998; Steimle et al., 1993; Waldburger et al., 2001, 2003). Induction of MHCII expression by IFN-γ is mediated by activation of the *CIITA* gene (Chang et al., 1994; Chin et al., 1994; Steimle et al., 1994; Waldburger et al., 2001). Repression of MHCII expression in plasma cells, trophoblasts, mature dendritic cells, and certain tumors is due to silencing of the CIITA gene (Holling et al., 2006; Landmann et al., 2001; Morris et al., 1998, 2000; Reith et al., 2005; Silacci et al., 1994). Cytokines that inhibit MHCII expression—such as TGFβ, IL-4, and IL-10—achieve this by inhibiting CIITA expression. Finally, a variety of pathogens have developed the ability to inhibit CIITA expression—and thus MHCII expression—as a strategy to evade recognition by the immune system (reviewed in LeibundGut-Landmann et al., 2004a). Examples include CMV, HIV, varicella-zoster virus, *Mycobacterium bovis*, *Mycobacterium tuberculosis, Toxoplasma gondii,* and *Chlamydia* (Abenroth et al., 2000; Kanazawa et al., 2000; Le Roy et al., 1999; Luder et al., 2003; Miller et al., 1998; Pai et al., 2003; Wojciechowski et al., 1999; Zhong et al., 1999).

Expression of the *CIITA* gene is directed by four independent promoters (I, II, III, and IV), which precede four alternative first exons (Fig. 16.2B). Three of these promoters (I, III, and IV) are conserved in the mouse *CIIta* gene. The *CIITA/CIIta* promoters differ in their cell-type specificity and response to IFN-γ (Fig. 16.2B). It is thus the differential activity of the *CIITA/CIIta* promoters that ultimately determines the cell-type specificity and inducibility of MHCII gene expression (reviewed in Harton and Ting, 2000; Reith and Mach, 2001; Reith et al., 2005; Ting and Trowsdale, 2002). Promoter I is used mainly in macrophages and most dendritic cells subsets (Irla et al., 2008; Landmann et al., 2001; LeibundGut-Landmann et al., 2004b; Muhlethaler-Mottet et al., 1997). Promoter III drives expression of CIITA in B cells, activated T cells, and a unique dendritic cell subset known as plasmacytoid dendritic cells (LeibundGut-Landmann et al., 2004b). Promoter IV is essential for IFN-γ–induced expression in non-bone-marrow–derived cells and for expression in cortical thymic epithelial cells (Irla et al., 2008; Muhlethaler-Mottet et al., 1998; Waldburger et al., 2001, 2003). Medullar thymic epithelial cells rely on both promoters III and IV (Irla et al., 2008).

Deregulation of the *CIITA* promoters is associated with diseases (reviewed in Holling et al., 2006; LeibundGut-Landmann et al., 2004a; Reith et al., 2005) (Fig. 16.2B). Both silencing and aberrant activation of promoters III and IV have been associated with various types of tumors. Several pathogens have developed mechanisms for avoiding immune recognition by inhibiting IFN-γ–induced activation of promoter IV.

FUNCTION AND MODE OF ACTION OF RFX

In contrast to CIITA, the RFX complex is expressed widely in all cell types examined, even in MHCII-negative cells. However, like CIITA, the MHCII, *Ii* (*CD74*), *HLA-DM*, and *HLA-DO* genes are the major target genes of RFX. RFX is also implicated in expression of MHCI and β2m genes (Gobin et al., 1998; van den Elsen et al., 1998). It is unlikely that RFX plays a major role in other systems, because all clinical and immunological features of RFX-deficient patients (groups B, C, and D) can be attributed to defects in MHC expression.

In vivo footprint experiments (Kara and Glimcher, 1991, 1993) and DNAse I hypersensitivity studies (Gonczy et al., 1989) have shown that MHCII promoters are completely unoccupied in RFX-deficient patients (Table 16.3, Fig. 16.1). This indicated that occupation of the promoter by DNA-binding factors such as the X2 box binding protein X2BP (Hasegawa and Boss, 1991), which contains CREB (Moreno et al., 1999), and the Y box binding protein NF-Y (Mantovani, 1999) is dependent on binding of RFX to the adjacent X box. An explanation for these findings was provided by in vitro binding studies demonstrating that RFX binds cooperatively with X2BP(CREB) and NF-Y to form a higher-order protein–DNA complex containing all three proteins (Fig. 16.2A) (Durand et al., 1994; Louis-Plence et al., 1997; Moreno et al., 1995; Reith et al., 1994a, 1994b). This nucleoprotein complex has been coined the MHCII "enhanceosome" complex (Masternak et al., 2000b). In the MHCII enhanceosome, the interactions of RFX, X2BP(CREB), and NF-Y with their respective target sites are strongly stabilized (Louis-Plence et al., 1997; Moreno et al., 1995; Reith et al., 1994a, 1994b). This stabilization is sufficiently strong to permit recruitment of RFX, X2BP(CREB), and NF-Y to all MHCII promoters, even to those that contain only very-low-affinity binding sites for these proteins (Louis-Plence et al., 1997; Reith et al., 1994a). These cooperative binding interactions are essential for stable occupation of MHCII promoters in vivo (Wright et al., 1994). The bare promoter phenotype observed in groups B, C, and D is thus a direct consequence of the deficiency in RFX (Fig. 16.1). The fact that the occupation of MHCII promoters requires cooperative binding interactions between RFX, X2BP(CREB),

and NF-Y also explains the observations that the order, orientation, and spacing of the X, X2, and Y sequences are highly conserved and critical for their activity (Reith et al., 1994b; Vilen et al., 1991, 1992).

One of the main functions of the enhanceosome is to serve as a platform for the recruitment of CIITA (Fig. 16.2A). However, there is growing evidence that the enhanceosome also plays several important CIITA-independent functions. First, at certain MHCII promoters, the enhanceosome is sufficient for inducing histone acetylation and recruitment of the general transcription machinery and RNA polymerase II (Masternak et al., 2002). Second, enhanceosome assembly plays a critical role in evicting nucleosomes from MHCII promoters, a process that is required for defining the correct position of the transcription initiation sites of MHCII genes (Leimgruber et al., 2009). Finally, the enhanceosome has recently been shown to protect MHCII genes against the establishment of epigenetic silencing by DNA methylation (Seguín-Estévez et al., 2009).

MUTATION ANALYSIS

Nine different mutations of the *CIITA* gene have been characterized (Fig. 16.3) (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a). These include two nonsense mutations, two missense mutations, a small in-frame deletion, and four splice-site mutations. These mutations all affect either the central segment of CIITA containing an NLS and the NOD domain, or the C-terminal region containing a second NLS and the LRR domain. One of the missense mutations was found to be responsible for a mild form of MHCII deficiency (Wiszniewski et al., 2001). An additional patient was found to exhibit a strong decrease in CIITA mRNA expression, which was attributed to a putative regulatory mutation in the CIITA gene (Dziembowska et al., 2002). Mutations affecting the two alleles of *CIITA* in the in vitro-generated RJ2.2.5 cell line have also been defined (Steimle et al., 1993).

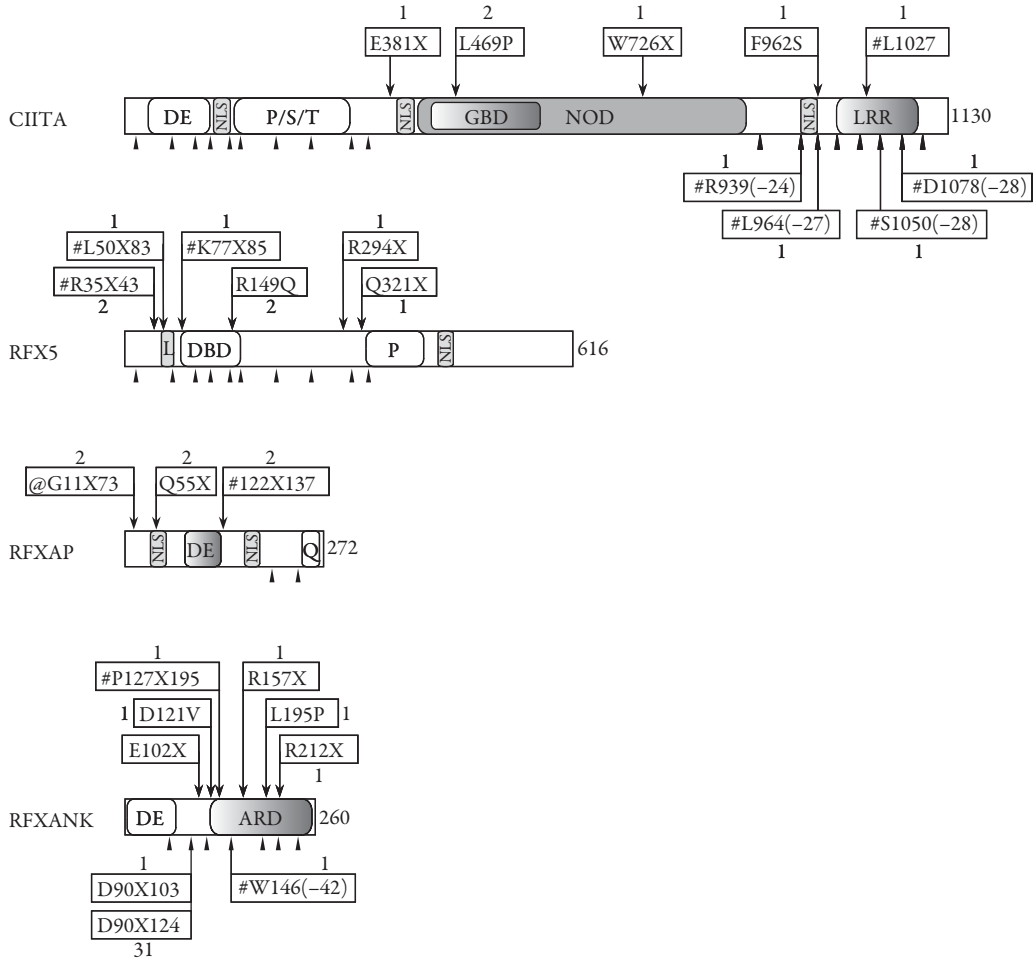

Figure 16.3 Structure and mutations of the factors that are affected in MHCII deficiency. Maps of CIITA, RFX5, RFXAP, and RFXANK are drawn to scale. The sizes in amino acids of the proteins are indicated at the right. Arrowheads below the maps indicate the positions of introns. Boxed text is used to describe mutations: boxes above the proteins correspond to mutations in coding regions, whereas boxes below correspond to mutations in splice sites. X denotes a stop codon. The number following an X indicates the position of a premature stop codon created by a frameshift. # indicates a deletion. @ indicates an insertion. Numbers in parentheses indicate the number of amino acids removed by an internal deletion. Numbers above or below the boxes indicate the number of patients in which the same mutation was found. Key protein domains are indicated: ARD, ankyrin repeat domain; DBD, DNA-binding domain; DE, rich in acidic amino acids (glutamic and aspartic acid); GBD, GTP-binding domain; L, leucine-rich stretch; LRR, leucine-rich repeats; NES, nuclear export signal; NLS, nuclear localization signal; NOD, nucleotide-binding and oligomerization domain; P, proline-rich; P/S/T, proline, serine, and threonine-rich; Q, glutamine-rich.

Nine different mutations of the *RFXANK* gene have been characterized (Fig. 16.3) (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a; Picard, unpublished data). These include three nonsense mutations, two missense mutations, a deletion leading to a frameshift, and three splice-site mutations. Eight of the mutations affect the integrity of the ankyrin repeat region, which is known to be essential for the function of RFXANK (Krawczyk et al., 2005; Nekrep et al., 2001). One is a missense mutation lying within the third ankyrin repeat. The remaining seven lead to the synthesis of proteins lacking all or part of the ankyrin repeat region. A 26 bp deletion disrupting a splice site has been found in 31 unrelated North African patients, indicating the existence of a founder effect (Wiszniewski et al., 2000; Picard, unpublished data). Another splice-site mutation was found to be responsible for a mild form of the disease (Prod'homme et al., 2003).

Six mutations of the *RFX5* gene have been identified (Fig. 16.3) (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a). These include three deletions leading to a frameshift, two nonsense mutations, and one missense mutation. The missense mutation lies in the DBD and was identified in two related patients exhibiting an atypically mild form of MHCII deficiency (Nekrep et al., 2002). This mutation does not abolish DNA-binding activity completely and therefore allows residual expression of certain MHCII genes. The remaining mutations all lead to the synthesis of severely truncated RFX5 proteins lacking the DBD and/or the C-terminal moiety of the protein. A mutation within RFX5 has also been identified in G1B, which is an in vitro-generated MHCII regulatory mutant. The RFX5 gene in G1B contains a missense mutation situated just upstream of the DNA binding domain (Brickey et al., 1999).

Three different mutations affecting the *RFXAP* gene have been identified (Fig. 16.3) (reviewed in Krawczyk and Reith, 2006; Masternak et al., 2000a). These include two frameshift mutations resulting from an insertion and a deletion, and a nonsense mutation. All of these mutations lead to the synthesis of severely truncated proteins lacking a C-terminal glutamine-rich region, which is known to be essential for the function of RFXAP. Frameshift mutations resulting from the insertion of G nucleotides in the *RFXAP* gene were also identified in the in vitro-generated 6.1.6 cell line (Durand et al., 1997). One patient was recently found to carry a 75 bp insertion in the 5' untranslated region of the *RFXAP* gene (van Eggermond et al., 2008). This insertion constitutes a regulatory mutation that abrogates *RFXAP* expression by impairing the activity of its promoter.

STRATEGIES FOR DIAGNOSIS

Early diagnosis is critical because it increases the chances of successful hematopoietic stem cell transplantation (HSCT). Young children presenting with clinical and immunological features typical of MHCII deficiency should be referred by their physicians to specialized centers as soon as possible. The decisive criteria for the diagnosis of MHCII deficiency are the absence of MHCII expression on monocytes and B cells. These parameters should be tested in children having recurrent upper respiratory tract infections, diarrhea, and failure to thrive. Additional criteria warranting an investigation of MHCII expression include low $CD4^+$ T-cell counts, hypogammaglobulinemia, and profoundly impaired antigen-specific T- and B-cell–mediated immune responses.

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

MHCII deficiency is inherited as an autosomal recessive disease. Carriers are healthy and exhibit no known phenotype. Considering the rarity of the disease, carrier detection is of no interest unless a consanguineous union is envisaged despite genetic counseling. In this case it could be performed either by using polymorphic markers flanking the affected gene or by a direct search for known mutations. Given the severity of MHCII deficiency, prenatal diagnosis in affected families is a valid ethical option. Previously, prenatal diagnosis has been carried out safely between the 20th and 22nd week of amenorrhea by analyzing MHCII expression on fetal leukocytes obtained by means of an umbilical vein puncture guided by echography (Durandy et al., 1987). However, characterization of the mutations affecting the *CIITA*, *RFXANK*, *RFX5*, and *RFXAP* genes in families from complementation groups A, B, C, and D now offers a better option for prenatal diagnosis. The presence of these mutations could be assessed directly on trophoblasts obtained from a chorionic villi biopsy during the 11th week of amenorrhea.

TREATMENT AND PROGNOSIS

Treatment of infections and other complications can at best reduce the frequency and severity of the clinical problems associated with MHCII deficiency. The optimal symptomatic care available consists of the prophylactic use of antibiotics and administration of immunoglobulins. However, these means do not prevent progressive organ dysfunction and death. In the absence of curative treatment (HSCT), the prognosis is poor. As indicated in Table 16.4, the majority of patients who do not undergo HSCT die at a young age from various infections, with or without autoimmune manifestations. Only a minority of patients characterized by a less severe clinical picture survive beyond the age of 20 years. There is no obvious difference in prognosis for patients belonging to the four different genetic complementation groups. The leaky phenotype characteristic of atypical patients is associated with a better outcome (Hauber et al., 1995; Prod'homme et al., 2003; Wiszniewski et al., 2001; Wolf et al., 1995).

As for other combined immunodeficiency disorders, HSCT is currently the only available curative treatment for MHCII deficiency. The outcome of HSCT in patients with MHCII deficiency is summarized in Table 16.4. The success rate is relatively poor, since less than half of the patients were cured by the procedure (Klein et al., 1995). Results were much better in patients undergoing HSCT before the age of 2 years,

Table 16.4 OUTCOME WITH AND WITHOUT HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

OUTCOME	*NO. PATIENTS*
Without HSCT	*21*
Pneumonia	20
Chronic diarrhea	19
Hepatitis, cholangitis	9
Sepsis	7
Meningoencephalitis	5
Autoimmunity	5
Alive, 1–31 years old (mean 10.5 years)	11
Death, 5 months to 16 years old (mean 5.7 years)	10
All HSCT	*39*
Alive 1–27 years after HSCT	16
Persistent immunodeficiency	1
Death after HSCT	23
HLA-Identical HSCT	*18*
Alive, 1–27 years after HSCT	10
Death, 46 to 384 days after HSCT	8

Data is from 60 patients treated at Hospital Necker-Enfants Malades between 1977 and 2009 (Renella et al. 2006; Picard, unpublished data).

probably because the viral burden and organ dysfunction are less intense (Klein et al., 1995). When an HLA-identical sibling is available, the chances of success are fairly good. Reduced survival after HLA-identical HSCT is caused by the high incidence of preexisting viral infections and is associated with the onset of severe acute graft-versus-host disease (Renella et al., 2006). The success rate is lower when HSCT is performed with matched unrelated donors or with partially HLA-compatible related donors. Although several patients have now been cured by HSCT with non–HLA-identical donors (Klein et al., 1995), the success rate remains lower than in other immunodeficiency syndromes. The two main obstacles are intractable persistent viral infections caused by a long-lasting T-cell immunodeficiency, and graft failure or rejection resulting from normal allogeneic responses in the patients.

Four conclusions can be drawn from our current experience with HSCT in MHCII deficiency (Klein et al., 1995; Renella et al., 2006). First, despite the lack of MHCII expression, the risk of graft-versus-host disease is similar to that observed in patients with other forms of immunodeficiency. Second, $CD4^+$ T-cell counts remain low (albeit functional) in long-term survivors because of defective MHCII expression by the thymic epithelial cells of the host. Third, the lack of MHCII expression in nonhematopoietic cells does not appear to be detrimental for patients who have undergone successful HSCT. Finally, given the invariably fatal course of typical MHCII deficiency and the poor outcome of HSCT performed after the age of 2 to 4 years, it is highly recommended that HSCT be performed in young children independently of whether or not an HLA-identical sibling is available.

Now that the four genes affected in MHCII deficiency have been identified, gene therapy has become a potential alternative to HSCT. Introduction of the wild-type *CIITA*, *RFXANK*, *RFX5*, or *RFXAP* genes into the hematopoietic stem cells of patients in complementation groups A, B, C, and D, respectively, would represent a logical therapeutic strategy. However, current gene therapy protocols remain hampered by the relative inefficiency of gene transfer into human hematopoietic stem cells, as well as by the risks entailed by vector insertion into the genome. Moreover, in normal individuals MHCII expression is tightly controlled in a cell-type–specific and inducible manner, and ectopic or nonphysiological levels of MHCII expression induced by the transgene should therefore be avoided. This should not represent a major problem for gene therapy with *RFX5*, *RFXANK*, and *RFXAP*, which are expressed ubiquitously at relatively constant levels in all cell types. Expression of CIITA is, on the other hand, tightly regulated. Correct expression of a *CIITA* transgene will be difficult to obtain unless the endogenous promoters of the *CIITA* gene are used.

MOUSE MODELS FOR MHCII DEFICIENCY

There are no spontaneous animal models for MHCII deficiency, but three mouse models have been constructed by gene targeting. The first model to become available was the MHCII knockout mouse, which reproduced many of the immunopathological features of the human disease, including hypogammaglobulinemia, decreased $CD4^+$ T-cell counts, and a deficiency in cellular and humoral immune responses to foreign antigens (reviewed in Dardell et al., 1994; Grusby and Glimcher, 1995). A more faithful model reproducing the molecular defect exhibited by patients in complementation group A was obtained by gene targeting of the mouse *CIIta* gene (Chang et al., 1996a; Itoh-Lindstrom et al., 1999; Williams et al., 1998). The phenotype exhibited by *CIIta* knockout mice is very similar to that of human MHCII-deficiency patients. Both constitutive and IFN-γ–induced MHCII expression is strongly reduced. There is also a drastic reduction in the number of $CD4^+$ T cells. $CD4^+$ T-cell–dependent immune responses are consequently severely compromised. A third model reproducing the molecular defect exhibited by patients in complementation group C was constructed by disruption of the mouse *Rfx5* gene (Clausen et al., 1998). The phenotype of *Rfx5* knockout mice is similar to that of *CIIta* knockout mice. Both constitutive and IFN-γ–inducible MHCII expression are lost, $CD4^+$ T-cell counts are strongly reduced, and adaptive immune responses are markedly impaired.

Both the *CIIta* and *Rfx5* knockout mice exhibit residual MHCII expression in certain tissues and cell types (reviewed in Reith and Mach, 2001), implying that there are RFX5- and/or CIITA-independent pathways for MHCII expression in specific cellular compartments. The precise pattern of residual expression differs between the two mice. $Rfx5^{-/-}$ mice retain MHCII expression in the thymic medulla and

significant, albeit weak, expression on a fraction of splenic and bone-marrow–derived dendritic cells, and on B cells activated in vitro with lipopolysaccharide (LPS) and/or IL-4 (Clausen et al., 1998). In contrast, residual MHCII expression in *CIIta*$^{-/-}$ mice concerns primarily dendritic cells in the lymph nodes, B cells in germinal centers, and a subset of thymic epithelial cells (Chang et al., 1996a; Williams et al., 1998). This difference in the pattern of residual MHCII expression is surprising because the human disease is phenotypically homogeneous. Leaky expression has been observed in cells from certain BLS patients, but no characteristic residual expression pattern distinguishing RFX-deficient patients from those with defects in CIITA has been described. This discrepancy could reflect species-specific differences in the respective roles of the MHCII regulatory factors. However, because of the rarity and severity of the disease, only relatively few patients from defined complementation groups have been studied in detail with respect to residual MHCII expression. Consequently, it is also possible that the phenotypic differences observed in the mouse system exist in the human disease as well but have escaped attention until now.

The development of CD4$^+$ T lymphocytes in the thymus requires positive selection driven by the expression of MHCII molecules on cortical thymic epithelial cells (Viret and Janeway, 1999). The fact that the CD4$^+$ T-cell population is almost completely absent in MHCII, *CIIta*, and *Rfx5* knockout mice is consistent with this (Chang et al., 1996a; Clausen et al., 1998; Dardell et al., 1994; Grusby and Glimcher, 1995). It is therefore quite surprising that CD4$^+$ T-cell counts are only mildly reduced in MHCII-deficiency patients (Griscelli et al., 1993; Klein et al., 1993). This discrepancy between MHCII deficiency and the existing mouse models for the human disease remains unresolved. One possible explanation is that mutations in the human *CIITA* or *RFX5* genes may permit a low residual level of MHCII expression on cortical epithelial cells of the thymus and that this residual expression is sufficient to drive positive selection of CD4$^+$ T thymocytes. In this context it may be relevant that residual MHCII expression in the thymus has indeed been described in certain patients (Griscelli et al., 1993; Schuurman et al., 1985). A subset of thymic epithelial cells retaining the ability to express MHCII molecules has also been described in *CIIta* knockout mice (Chang et al., 1996a) and in *Rfx5* knockout mice (Clausen et al., 1998). An alternative explanation could be that CD4$^+$ T-cell selection in humans can be driven by alternative mechanisms that differ from those most prominent in the mouse. Interestingly, an analysis of the CD4$^+$ T-cell repertoire in MHCII-deficiency patients has revealed subtle alterations suggesting that CD4$^+$ T cells in these patients may have escaped the normal thymic selection processes (Henwood et al., 1996). It should be mentioned, however, that the same alterations were not observed in another study (Rieux Laucat et al., 1993).

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17.

PEPTIDE TRANSPORTER DEFECTS IN HUMAN LEUKOCYTE ANTIGEN CLASS I DEFICIENCY

Henri de la Salle, Lionel Donato, and Daniel Hanau

Human leukocyte antigen (HLA) class I molecules present peptides, derived from proteins synthesized in the cell, to cytotoxic αβ $CD8^+$ T lymphocytes. In this way, they are involved in immune responses against intracellular pathogens and cancer cells. Although HLA class I molecules should a priori be essential to immune defense and thus to survival, a few cases have been described of children or adults who live with a low level of HLA class I cell surface expression. Notably, complete HLA class I deficiency has never been reported, which suggests that total deficiency would be lethal. The first case of HLA class I deficiency, or bare lymphocyte syndrome (BLS), was identified at a time when HLA class II typing techniques were unreliable. This case was later found to be an HLA class II deficiency associated with low expression of class I molecules (BLS type III) (Sabatier et al., 1996; Touraine et al., 1978), resulting from a defect in the RFXAP transcription factor (Durand et al., 1997). Two cases of class I deficiency (BLS type I) without immunodeficiency were subsequently described in a nonconsanguineous family (Payne et al., 1983). One was discovered when bone marrow transplantation was being considered to treat aplastic anemia, while the second was a younger brother who displayed the same defect of HLA class I expression but was healthy. Four other cases of BLS type I were identified in patients who had first-cousin parents and suffered from unexplained lung disease (de la Salle et al., 1994; Maeda et al., 1985; Sugiyama et al., 1989). Apart from one who experienced recurrent fever (Sugiyama et al., 1989), these patients did not seem to be abnormally susceptible to viral infections. Moreover, when complete clinical data were available, the patients often appeared to have been healthy during the first years of life. Thus, the pathology associated with BLS type I does not appear to be a direct consequence of the deficiency (i.e., an expected susceptibility to viral infections or cancer) but rather to result from secondary effects. Due to this absence of a direct link between clinical manifestations and deficiency, the discovery of the first cases of BLS type I was fortuitous, subsequent to serological HLA typing, by clinicians faced with a desperate situation. Since the first molecular genetic and clinical descriptions of two patients in 1994, other HLA class I-deficient individuals have been discovered among previous and new clinical cases.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Only 28 well-documented cases of HLA class I deficiency with normal expression of class II molecules have been published, 23 of them resulting from a defect in the peptide transporter associated with antigen processing (TAP). Contrary to BLS types II and III, which are characterized by decreased HLA class II expression and the early onset of severe combined immunodeficiency, HLA class I deficiencies do not lead to any particular pathological manifestations during the first years of life. Pathology of the gut (diarrhea) is not observed, unlike in BLS types II or III. The clinical course of the known cases (reviewed in Gadola et al., 2000 and summarized in Table 17.1) varies among individuals. Chronic inflammatory lung disease, developing generally in late childhood, is the most common, but not requisite, trait of TAP-deficient patients. It begins with infections with *Haemophilus influenzae* during the first decade of life, is confined to the respiratory tract, and extends from the upper to the lower airways. Other bacterial pathogens may also be found (*Streptoccocus pneumoniae*, *Klebsiella*, *Pseudomonas aeruginosa*). The high frequency of nasosinusal involvement and nasal polyposis, uncommon in children who do not have cystic fibrosis, is noteworthy.

Table 17.1 CHARACTERISTICS OF HLA CLASS I DEFICIENCIES CAUSED BY MUTATIONS IN TAP1 AND TAP2

SITE OF CLINICAL DISEASE	HLA HAPLOTYPE	MOLECULAR DEFECT	REFERENCE
RT	unknown	unknown	1
RT, skin	A*2402 B*4006 Cw*15 DRB1*08032 DPB1*0501 DQB1*0601	TAP1(260)	2
RT	A*0301B*1516Cw*1402 DRB1*0405 DRB4*0101 DQB1*0301 DPA1*0301 DPB1*0302	TAP2(273)	3
RT	A*0301B*1516Cw*1402 DRB1*0405 DRB4*0101 DQB1*0301 DPA1*0301 DPB1*0302	TAP2(273)	3
RT, Skin	A*2601 B*4901Cw*07 DRB1*1302 DRB3*0301 DPB1*1501 DQB1*0604	TAP1(274)	4
RT, skin	A*2601 B*4901Cw*07 DRB1*1302 DRB3*0301 DPB1*1501 DQB1*0604	TAP1(274)	5
RT, skin	A*2601 B*4901Cw*07DRB1*1302 DRB3*0301 DPB1*1501 DQB1*0604	TAP1(274)	6
RT	A*03 B*1501 Cw*03 DR*1301 DQ*0603	TAP1	4
Skin	A*2301 B*4901Cw*0701 DRB1*0301 DQB1*0201	TAP1	4, 7
RT, skin	A*11 B*1502 Cw0801 DRB1*15 DQB1*0601	TAP2(326)	4, 8
RT, skin	A*11 B*1502 Cw0801 DRB1*15 DQB1*0601	TAP2(326)	4
RT	A*01 B*51 Cw*07 DRB1* 1305 DRB3*0202 or DQB1*03011	TAP2(220)	9
Minor skin granuloma	A*0301 B*07021 Cw*0702 DRB*15 DQB1*0106	TAP2(546)	10
Asymptomatic	A*0301 B*07021 Cw*0702 DRB*15 DQB1*0106	TAP2(546)	10
RT	A*0301 B*07021 Cw*0702 DRB*15 DQB1*0106	TAP2	11
RT	A*0301 B*07021 Cw*0702 DRB*15 DQB1*0106	TAP2	11
RT	A*0301 B*07021 Cw*0702 DRB*15 DQB1*0106	TAP2	11
Ocular toxoplasmosis	A*24 B*14 DRB1*13 DQB1*06	TAP1(582)	12
Asymptomatic	A*24 B*14 DRB1*13 DQB1*06	TAP1(582)	12
RT, skin	A*26 B*38 DRB1*03 DQB1*02	TAP1(438)	13
RT, skin, pulmonary toxoplasmosis	A*26 B*38 DRB1*03 DQB1*02	TAP1(438)	13
RT, skin	A*26 B*08 Cw*7 DRB1*15 DQB1*05	TAP2(340)	14
RT, skin	A*01 B*08 Cw7 DRB1*0701 DQB1*02	TAP2(623)	15
RT, skin	A*01 B*08 Cw*7 DR17 DR52 DQ2	TAP1(747)	16

RT, Respiratory tract disease including, depending on the case and the description, recurrent sinusitis, bronchitis, colonization with bacteria (*H. influenzae, P. aeruginosa*), obstructive impairment, bronchiectasis and emphysema. Skin: skin granulomas. Although uncharacterized, patient 1 displayed a typical TAP-deficient syndrome and was therefore included in the table. All the HLA haplotypes identified were homozygous.

Cases belonging to the same family are grouped; thus, 15 families, representing 23 confirmed TAP-deficient patients, have been described. Unrelated familial cases originating from the same geographical area and sharing the same HLA haplotypes are highlighted in gray. In the defect column, the positions of the mutated codons are given in parentheses.

References in which the mutations are described: 1, Sugiyama et al., 1989; 2, Azuma et al., 2001; de la Salle et al., 1999; Furukawa et al., 1999a, 1999b, 1999c, 2000; Maeda et al., 1985; Sugiyama et al., 1986; Watanabe et al., 1987; 3, de la Salle et al., 1994; Donato et al., 1995; Zimmer et al., 1998, 1999, 2007; 4, Moins-Teisserenc et al., 1999; 5, Caversaccio et al., 2008; 6, de la Salle et al., 1999; Plebani et al., 1996; 7, Willemsen et al., 1995; 8, Teisserenc et al., 1997; 9, Matamoros et al., 2001; 10, de la Salle et al., 2002; 11, Parissiadis et al., 2005; 12, Markel et al., 2004a, 2004b; 13, Dogu et al., 2006; 14, de la Salle et al., 2007a; 15, de la Salle et al., 2007b; 16, Villa-Forte et al., 2008.

The pathology inevitably evolves to a respiratory insufficiency resulting in bronchiectasis, emphysema, panbronchiolitis, or bronchial obstruction. In the early stages of the disease, computed tomography (CT) scans of the chest may show bronchiolectasis, which later leads to bronchiectasis (Fig. 17.1). Hypoxemia can occur during infectious exacerbation.

Skin lesions have been described in several patients and start with local inflammation, most often on the legs, developing into necrotizing granulomas. These lesions may be related to vasculitis, leading to an inappropriate diagnosis of Wegener's granulomatosis (Plebani et al., 1996; Teisserenc et al., 1997; Watanabe et al., 1987; Villa-Forte et al., 2008). In a number of cases, lesions occurring in the upper respiratory tract led to mutilation of the midface (Moins-Teisserenc et al., 1999). One of these patients did not display inflammation of the airways. Surprisingly, the defect may remain unnoticed for many years. Thus, two adults without inflammatory lung disease have been described, one totally asymptomatic and the other with minor skin lesions on one leg (de la Salle et al., 2002). These lesions generally heal only transiently and

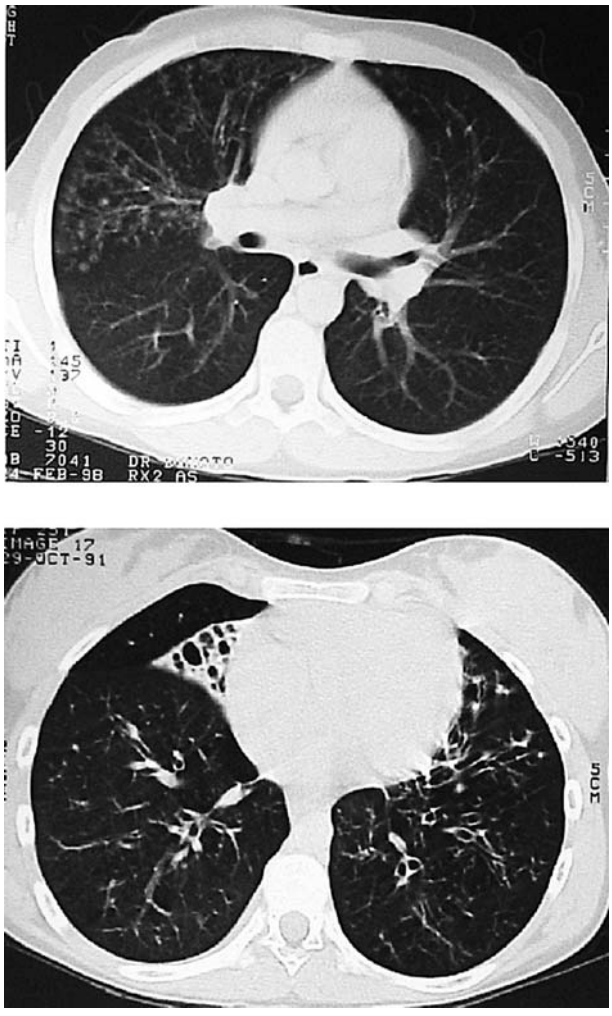

Figure 17.1 Chest CT scans. (Top) Bronchiolectasis in a 15-year-old TAP2-deficient patient. Patchy opacities spread out along the bronchovascular axes within the right middle lobe. (Bottom) Bilateral bronchiectasis in the patient's eldest sister, also TAP2 deficient. The lesions extend to the right middle lobe, lingula, and left lower lobe and represent a later stage of disease.

relapse, so they are difficult to treat and represent a major as yet unsolved dermatological problem.

Susceptibility to intracellular pathogens would be expected; accordingly, toxoplasmosis has been documented in TAP-deficient patients. In a 15-year-old patient, ocular toxoplasmosis resulted in necrotizing retinochoroiditis, which, despite anti-Toxoplasma therapy and subsequent surgery, led to the loss of one eye (Parissiadis et al., 2005). Another patient was treated for pulmonary toxoplasmosis; the clinical history revealed that there had been loss of vision in one eye for unknown reasons a few months before, which suggests that the toxoplasmosis might have initiated with an ocular infection (Dogu et al., 2006). Finally, poorly controlled pulmonary tuberculosis could be observed in a TAP-deficient child in Portugal having the same mutation than that of a Brasilian patient (unpublished observations, Villa-Forte et al., 2008).

An absence of efficient HLA class I restricted immune responses might finally lead to susceptibility to cancer. One case has been documented do date, a recent observation of skin carcinoma initiating within a skin lesion (España et al., 2010). This is interesting in that it raises the question as to whether the cancer resulted from an infection with a pathogen such as Merkel polyomavirus, which could not be investigated due to a lack of material.

The two HLA class I-deficient brothers described by Payne et al. were free of lung and upper airway disease, although respiratory involvement could not be excluded because the patients were young, no ear-nose-throat or pulmonary investigations had been carried out, and long-term data were lacking. One of these children suffered from unexplained anemia but recovered under prolonged corticosteroid treatment. Another case has since been discovered in France (de la Salle, unpublished data). As shown in the next section, this type of deficiency is not caused by a TAP defect.

Finally, a moderate HLA class I deficiency resulting from a defect in tapasin (TAP-associated glycoprotein) has been characterized. The patient did not display any of the symptoms associated with TAP deficiency but suffered from chronic primary glomerulonephritis (Yabe et al., 2002).

MOLECULAR BASIS OF TAP DEFICIENCY

MECHANISMS OF ANTIGEN PRESENTATION BY HLA CLASS I MOLECULES

Classical HLA class I molecules are composed of a polymorphic membrane-associated heavy chain, encoded by HLA-A, HLA-B, and HLA-C genes, associated with β2-microglobulin (β2m), a soluble subunit. After assembly in the endoplasmic reticulum, the heavy chain/β2m complexes associate with calreticulin, TAP, tapasin, and ERp57, forming altogether the "peptide loading complex" (PLC, see Fig. 17.2) (Wearsch and Cresswell, 2008). Calreticulin is a chaperone that controls the quality of proteins assembled in the endoplasmic reticulum, here newly synthesized heavy chain/β2m heterodimers. Within the PLC, TAP imports into the lumen of the endoplasmic reticulum peptides resulting from the cytosolic degradation of proteins, mediated in general by the proteasome or in rare cases by tripeptidyl peptidase II (Kloetzel, 2004). TAP is composed of two subunits, TAP1 and TAP2, and belongs to the ATP binding cassette (ABC) family of transporters. The cytoplasmic region of these transporters includes two ATP binding cassettes (Walker sites A and B) necessary for their activity (Schneider and Hunke, 1998). However, in the case of TAP, the ATP binding site of TAP1 is dispensable (Karttunen et al., 2001). The first four and three transmembrane domains of TAP1 and TAP2, respectively, bind tapasin and are dispensable for the peptide transporter activity of TAP. The six other transmembrane segments and the C-terminal cytoplasmic domains of each subunit constitute the core domains necessary and sufficient for the assembly of TAP and its peptide transport activity (Koch et al., 2004). Tapasin mediates a physical link between TAP and the heavy chain/β2m heterodimer and favors the binding of optimal peptides. The function of

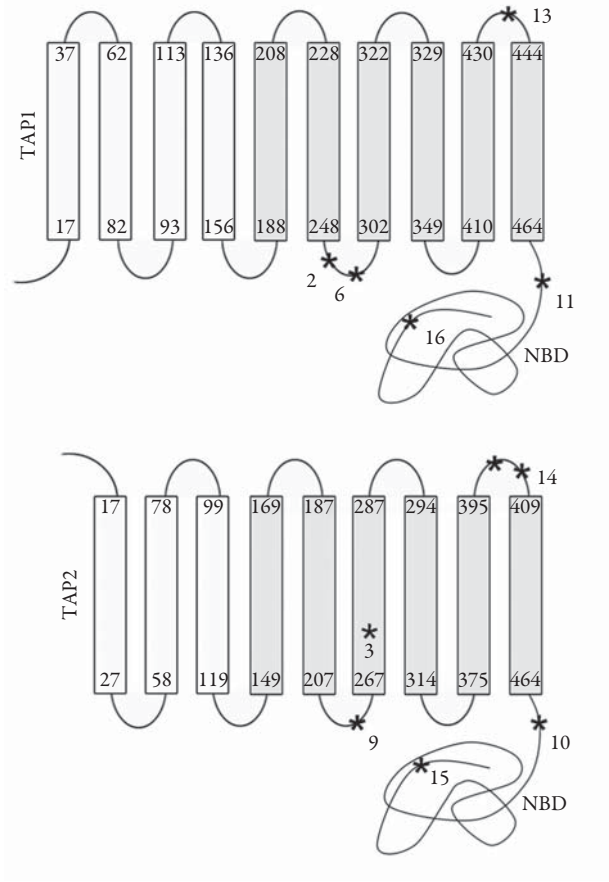

Figure 17.2 Structure of the TAP1 and TAP2 proteins and positions of the amino acids that have been found mutated in patients. The white and gray rectangles represent transmembrane domains respectively participating in interaction with tapasin, or constitutive to the transporter function. The numbers in the rectangles correspond to the amino acid coordinates of the N- and C-terminal ends of the domains (Koch et al., 2004). The mutations are numbered according to Table 17.1. NBD, nucleotide binding domain.

tapasin is controlled by ERp57, which associates with tapasin via a disulfide bridge. ERp57 increases the stability of the PLC and thereby facilitates the binding of optimal peptides by the heavy chain/β2m complex. ERp57 does not need to interact with calnexin or calreticulin to promote the assembly of class I histocompatibility molecules, and it enhances peptide loading independently of its redox activity (Zhang et al., 2009). Finally, the imported peptides may be too long to bind to HLA class I molecules, and hence the N-terminal ends of antigenic peptides may need to be processed by endoplasmic reticulum aminopeptidases associated with antigen processing (ERAP1 and ERAP2) (Wearsch and Cresswell, 2008).

Studies in tumor and mutant cell lines have shown that defective HLA class I expression may arise from downregulation of HLA class I or TAP genes, or from mutations in β2m, TAP, or tapasin genes. In TAP-deficient cell lines, most HLA class I molecules remain blocked between the endoplasmic reticulum and the cis-Golgi compartment and therefore stay endoglycosidase H sensitive and unsialylated. The HLA class I heavy chain/β2m complexes are peptide-free and unstable at 37°C, resulting in poor expression of HLA class I molecules on the cell surface. In the absence of TAP, the presentation of most peptides of intracellular origin is blocked, although TAP-independent presentation of some antigens can occur. The genes encoding the TAP subunits are located in the HLA class II genetic locus on chromosome 6 and are polymorphic; up to six TAP1 and four TAP2 human alleles have been described to date (Robinson et al., 2003). Calnexin is not essential for normal expression of HLA class I molecules (Scott and Dawson, 1995). In contrast, the processing of these molecules is altered in the tapasin-defective 721.220 cell line, in a manner similar to that in TAP-deficient cells, although the effect of the mutation depends on the biophysical characteristics of the HLA variants (Greenwood et al., 1994). Disruption of the ERp57 gene in mouse B lymphocytes affects the assembly of the PLC, leading to a moderate to weak decrease in plasma membrane MHC class I molecules, depending on the isoform, and a reduction of antigen presentation (Garbi et al., 2006). In the mouse, targeted disruption of the gene encoding the endoplasmic reticulum aminopeptidase had only a moderate effect on MHC class I expression on the plasma membrane, although antigen presentation could be significantly affected (Yan et al., 2006).

MOLECULAR BASIS OF HLA CLASS I DEFICIENCIES

Up until now, three kinds of HLA class I deficiency have been encountered. In two individuals (Payne et al., 1983), genetic analyses demonstrated that HLA class I genes were constitutively poorly expressed in lymphocytes (Sullivan et al., 1985). This deficiency was probably not linked to chromosome 6, as the two brothers had inherited different HLA haplotypes, but the causative genetic defect has not yet been elucidated. A second type of HLA class I deficiency arose from a tapasin defect. Only one patient has been described, born to consanguineous parents, HLA homozygous and bearing a homozygous mutation in the tapasin gene, located in the HLA region. The expression of HLA class I molecules was ten-fold reduced (Yabe et al., 2002), but no further biochemical or immunological investigations have been reported.

The third type is apparently the most common, probably because it generally leads to typical clinical manifestations. In all but one report, the patients were born to consanguineous parents, and when HLA typing could be performed, the individuals were always found to be HLA homozygous. In agreement with the location of TAP genes in the HLA class II genetic region, a homozygous mutation in one of the TAP genes was found in all characterized cases (Table 17.1). Biochemical studies of cell lines derived from these patients showed that their HLA class I heavy chains remained unsialylated (de la Salle et al., 1994, 1999; Maeda et al., 1985). Most of the heavy chains remained endoglycosidase H sensitive, while HLA class I molecules were unstable at 37°C (de la Salle et al., 1994, 1999, 2002; Teisserenc et al., 1997). Five mutations have been characterized in the TAP1 and six in the TAP2 gene; the MIM (Mendelian Inheritance in Man) numbers for

these deficiencies are 170260 and 170261, respectively. The main clinical features and the HLA haplotypes of the different patients are provided in Table 17.1. Identical HLA haplotypes were observed in different families, who appeared to originate from the same geographical areas. All the mutations generate subunits truncated in one of the transmembrane helices or in the cytoplasmic domain. Mutation in TAP1 may lead to instability of the TAP2 protein (Furukawa et al., 1999a; Moins-Teisserenc et al., 1999).

FUNCTIONAL ASPECTS

EXPRESSION OF HLA CLASS I MOLECULES

The expression of HLA class I molecules can be quantified by flow cytometry after staining of the cells with a pan-anti-HLA class I monoclonal antibody such as W6/32. On peripheral blood mononuclear cells (PBMCs) of the two individuals described by Payne et al., the expression of HLA class I molecules was reduced ten-fold, whereas this expression was normal on platelets (Sullivan et al., 1985). The latter observation would appear to contradict the fact that HLA typing could not be performed on platelets (Payne et al., 1983). Epstein-Barr virus-transformed B (EBV B) cell lines derived from the patients progressively expressed higher levels of HLA class I molecules after a few weeks of culture. In an analogous case, we observed that the PBMCs of a 2-year-old child displayed low HLA class I expression while an EBV B-cell line gradually expressed higher levels of these molecules, although still only 10 percent that of class I on normal EBV B cells. Additional experiments demonstrated that HLA class I genes could be induced in activated T cells, their expression being upregulated by tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α) or IFN-γ (de la Salle, unpublished observations). Thus, in this type of deficiency, HLA class I genes can be induced by inflammatory cytokines. As a result, HLA class I-mediated immune responses can be increased when necessary and, for this reason, the defect may be qualified as "conditional." Although the HLA class I expression in other tissues of these individuals was not documented, it is noteworthy that the expression of HLA class II molecules was normal.

On the surface of cells from TAP-deficient patients, the expression of HLA class I molecules is reduced 100-fold (de la Salle et al., 1994, 1999; Teisserenc et al., 1997) and the same level of reduction is found on lymphocytes, monocytes, neutrophils, and skin fibroblasts. This expression increased slightly on lymphocytes and fibroblasts after treatment with IFN-α or IFN-γ, whereas TNF-α induced weak upregulation on fibroblasts only. However, these observations reflected merely the capacity of these cytokines to upregulate HLA class I genes. A similar effect was noted when the PBMCs of another patient were incubated with phytohemagglutinin (PHA) and IFN-γ (Plebani et al., 1996). Two adults with a TAP2 deficiency were described who surprisingly expressed five times more HLA class I molecules than other TAP-deficient patients (de la Salle et al., 2002). This discrepancy was found to result from a moderate TAP-independent cell-surface expression of HLA-B7 molecules, which was 20 percent of that on normal HLA-B7 cells.

HLA class I molecules were found to be absent from the skin of TAP-deficient patients (Hanau et al., 1994). Immunohistochemical studies of skin from two other patients showed that these molecules were not expressed in healthy skin biopsies but were present in skin lesions. In one case, HLA class I expression was lower than in skin from normal individuals (Watanabe et al., 1987), while in the other the location of these molecules within cells or at the cell surface was uncertain (Plebani et al., 1996).

Soluble HLA class I molecules circulate in plasma. TAP-deficient patients and the child described above who displayed a low but inducible HLA class I expression on lymphocytes had very reduced plasma levels of soluble HLA class I molecules of 0.15 and 0.31 μg/mL, respectively (normal values, 160 ng to 3 μg/mL) (unpublished data).

EXPRESSION OF NONCLASSICAL HLA CLASS I MOLECULES

Nonclassical HLA class I molecules are composed of nonpolymorphic heavy chains associated with β2m and, unlike the ubiquitous classical HLA class I molecules, are expressed only on specialized cells. In particular, CD1 molecules are expressed on dendritic cells, including epidermal Langerhans cells. In two TAP-deficient patients, immunocytochemical staining of skin biopsies showed HLA class I molecules to be undetectable but CD1a antigens to be normally expressed on Langerhans cells. Flow cytometry further demonstrated that CD1 antigens were present at normal levels on dendritic cells derived in vitro from monocytes (Hanau et al., 1994). In another case, CD1a molecules were likewise normally expressed on epidermal Langerhans cells in biopsies taken from nonlesional skin but were not detectable in biopsies taken from ulcers (Plebani et al., 1996). This absence of CD1a from lesion biopsies might nevertheless result only from the local inflammation and not be linked to the genetic defect.

Another important HLA class I-like molecule is HLA-E, which presents, in a TAP-dependent manner, peptides derived from the signal peptides of classical HLA class I molecules. This protein interacts with the NKG-2A/CD94 inhibitory receptor expressed on natural killer (NK) cells and a subset of T cells and thereby acts as a sensor in the biosynthetic pathway of HLA class I molecules. HLA-E/NKG-2A/CD94 interactions suppress the cytolytic responses of NK and T cells and their cytokine release. Surprisingly, immunofluorescence studies showed HLA-E to be expressed on the PHA-induced T-cell blasts but not on the EBV B cells of a TAP-deficient patient (Furukawa et al., 1999b). However, in another study, HLA-E molecules were nearly undetectable on the surface of PBMCs and EBV B cells (Matamoros et al., 2001).

HUMORAL IMMUNE RESPONSE

The effects of HLA class I deficiency on the levels of immunoglobulin (Ig) classes and subclasses have been reported in a few

studies. Hypergammaglobulinemia with low levels of IgG2 and an absence of IgG4 was observed in one case (Plebani et al., 1996) and IgG2 deficiency in another (Matamoros et al., 2001).

Titration of antibodies in the sera of two TAP2-deficient patients showed them to have been infected by most common viruses: measles, mumps, herpes, cytomegalovirus, influenza, varicella, and EBV (Donato et al., 1995). Since these viral infections did not lead to an exaggerated pathology or require special care, the TAP-independent immune responses of these individuals must be to some extent efficient. This contrasts with the pathologies of herpes, cytomegalovirus, and EBV observed in patients lacking NK cells (Biron et al., 1989) or with diminished NK-cell activity (Caligiuri et al., 1987). The fact that TAP-deficient patients can have high titers of antibodies against measles, mumps, herpes, cytomegalovirus, or varicella suggests that antibodies are important in their defense against these viruses. High levels of antiviral antibodies have not, however, been reported in all patients. Moreover, the titers of antibodies against EBV, respiratory syncytial virus, and influenza were normal (Donato et al., 1995, and unpublished data), which would suggest that cell-mediated immune responses may be effective against viruses. In contrast, vaccinations failed to induce antibody responses to polysaccharides (Matamoros et al., 2001). Antibodies against herpes and EBV were detected in serum from one case of "conditional" HLA class I deficiency (unpublished observations).

BACTERICIDAL/PERMEABILITY INCREASING (BPI) PROTEIN-MEDIATED RESPONSES

BPI is a protein expressed by neutrophils and to lesser extent by eosinophils, epithelia, and fibroblasts (Schultz, 2007). This protein protects the organism from gram-negative bacteria through an antibiotic activity and through endotoxin detoxification. In a number of chronic bacterial infections, in particular in TAP-deficient patients with chronic bacterial colonization of the lung, anti-BPI antibodies develop (antineutrophil cytoplasmic autoantibodies, or ANCA). These antibodies neutralize the activity of BPI and may exacerbate the inflammation of the respiratory tract (Schultz et al., 2003).

T-LYMPHOCYTE SUBPOPULATIONS

Experiments in transgenic mice have shown that the positive selection of $CD8^+$ $\alpha\beta$ T cells is dependent on interaction of their T-cell receptors with HLA class I molecules expressed on thymic epithelial cells. Consequently, MHC class I-deficient mice have very low numbers of $CD8^+$ T cells. Most analyses of T-cell subpopulations in BLS type I patients have been limited to determination of the ratio of $CD8^+$ to $CD4^+$ T cells. This ratio frequently appears to lie within normal values (Maeda et al., 1985; Moins-Teisserenc et al., 1999; Plebani et al., 1996; Sugiyama et al., 1989; Teisserenc et al., 1997), although the absolute number of T cells decreased progressively in one case (Plebani et al., 1996). No data are available on T-cell subsets in the patients described by Payne et al. In the apparently similar case identified in our laboratory, the numbers of $CD4^+$ and $CD8^+$ $\alpha\beta$ T lymphocytes were normal (unpublished observations).

T-cell subpopulations have been analyzed in PBMCs from few TAP-deficient patients (de la Salle et al., 1994, 2002; Moins-Teisserenc et al., 1999). The ratio of $CD8^+$ to $CD4^+$ T cells may appear to be somewhat diminished as compared to that in normal individuals, while expansion $\gamma\delta$ T cells is noticed. In one patient who was considered at the time to be healthy, the ratio of $CD8^+$ cells among $\alpha\beta$ T cells in PBMCs was very low (4 percent) whereas the number of $\gamma\delta$ T cells was normal (8 percent of T cells). He also developed lung pathology, and during the progression of the disease, an expansion of $\alpha\beta$ $CD8^+$ (16 percent of $\alpha\beta$ T cells) and $\gamma\delta$ T cells was observed (14 percent of T cells), suggesting that these two subsets can be recruited in immune responses (de la Salle et al., 1994, and unpublished observations). An expansion of $\gamma\delta$ T cells has been observed in several but not all cases (de la Salle et al., 1999; Matamoros et al., 2001; Moins-Teisserenc et al., 1999). Thus, in type I BLS patients a large proportion of $CD8^+$ cells may correspond to HLA-unrestricted $\gamma\delta$ T cells; consequently, a relevant assessment and interpretation of $CD8^+$ T-cell frequencies and $CD8^+$ to $CD4^+$ cell ratios is possible only by analyzing exclusively $\alpha\beta$ T cells. On this basis, numbers of $CD8^+$ $\alpha\beta$ T cells appear to be generally decreased in these patients, although they sometimes remain within lower normal limits. Finally, the repertoire of $CD8^+$ $\alpha\beta$ T cells is oligoclonal and quite similar to that of $CD4^+$ cells (de la Salle et al., 1997). This suggests that the deficiency has only a weak influence on the $CD8^+$ T-cell repertoire.

NK LYMPHOCYTE SUBPOPULATIONS

The number of NK cells seems to stay within normal ranges in HLA class I-deficient patients, varying from lower to higher values, depending on the individual (de la Salle et al., 1994, 2002; Furukawa et al., 1999a; Matamoros et al., 2001; Moins-Teisserenc et al., 1999). A study of the phenotype of NK cells in two TAP2-deficient patients showed that these cells express the type III receptor for IgG (CD16) and the CD56 adhesion molecule. In addition, 40 to 50 percent of NK cells were found to express high levels of CD56, compared to less than 10 percent in normal subjects (Zimmer et al., 2007). In TAP^+ individuals, these cells have to be activated by IL-2 to become cytotoxic.

The cytolytic activity of NK cells is controlled by a balance of activating and inhibitory receptors (Moretta et al., 2000), the latter receptors blocking the cytolytic process when they interact with HLA class I molecules on the target cells. Resting TAP-deficient NK cells display a normally diverse repertoire of inhibitory receptors (Furukawa et al., 1999c; Zimmer et al., 1998). The levels of expression of these receptors are similar to those on normal cells, except for the NKG-2A/CD94 and ILT2 receptors, which were found to be overexpressed on NK cells but not on T cells in three cases (Matamoros et al., 2001; Zimmer et al., 1998, 2007). This overexpression of NKG-2A/CD94 was not found in another study (Furukawa et al., 1999b), while in the two patients from the first study, it seemed to decrease with age or improvement of the clinical status (unpublished observations). The inhibitory receptors

negatively regulate the cytolytic activity of TAP$^-$ NK cells and are therefore functional.

In a more recent study (Markel et al., 2004b), the repertoire of inhibitory receptors on NK clones derived from three patients from the same family was compared to that on NK clones from an unaffected sister. The number of clones expressing higher levels of inhibitory receptors was increased among TAP$^-$ clones. Interestingly, whereas TAP$^+$ NK cells rarely express the inhibitory carcinoembryonic antigen-related cell adhesion molecule 1 (CEA-CAM1), most of the TAP$^-$ clones expressed this molecule. This work showed that the repertoire of inhibitory receptors on the activated NK cells of these patients is unique.

The repertoire of most activating receptors is also normal on peripheral TAP$^-$ NK cells (Vitale et al., 2002). Thus, these cells express receptors involved in the killing of tumor cells (NKp30, 40, and 46) or virally infected cells (NKG2-D). On polyclonal NK cell lines derived from TAP-deficient patients, the repertoire of activating receptors nevertheless displayed some abnormality, since the expression of NKp80 was low on many cells. Moreover, the same abnormality is observed at a clonal level. Triggering of these receptors induced normal cytolytic responses, except for 2B4 and NKp80, which were found to be functional only on a limited number of NK clones. Hence, the deficiency appears to have an observable effect on a restricted spectrum of NK receptors and only in some cells.

An interesting feature of NK cells from patients with chronic inflammation is the expression of CCR2. Bronchoalveolar fluids contain relatively large amounts of CCR2 ligand MCP1 and IL-2, which can activate recruited NK cells (Hanna et al., 2005). These observations support the hypothesis of a role of activated NK cells in the pathology of TAP-deficient patients (Moins-Teisserenc et al., 1999; Zimmer et al., 1998).

CELL-MEDIATED IMMUNE RESPONSES

The cytotoxicity of TAP-deficient NK and T cells has been investigated in several patients. Although resting NK cells did not appear to kill the HLA class I-negative K562 cells classically used in NK cytotoxicity assays, they were able to mediate a weak antibody-dependent cytotoxicity. After activation in vitro with IL-2 or IL-12, these NK cells killed K562 cells but not other HLA class I-negative cells such as Daudi or 721.221 cells (Furukawa et al., 1999c). When TAP-deficient NK cells were stimulated with EBV B cells and IL-2, they proliferated and developed cytotoxicity against several target cells in the same manner as NK cells from normal donors, although with less efficiency. More importantly, these activated TAP-deficient NK cells were cytotoxic to autologous EBV B cells and skin fibroblasts. Cytokines, which increase HLA class I expression and consequently protect normal fibroblasts from lysis by control activated autologous NK cells, did not protect against TAP-deficient cells (Zimmer et al., 1999). Reactivity to autologous EBV B cells and fibroblasts is mediated by an NK-cell subset expressing high levels of NKp46 (Vitale et al., 2002). Thus, it would appear that in vivo the NK cells of TAP-deficient patients can be activated by cytokines released during antiviral responses, and that their activated NKp46 bright NK cells can be aggressive to the surrounding uninfected cells.

In contrast, activated TAP$^-$ NK cells do not kill autologous T-cell blasts, whereas fewer than 10 percent of TAP$^+$ NK cell clones are unable to kill TAP$^-$ T-cell blasts. This tolerance to autologous T cells in TAP-deficient patients could be mediated by an inhibitory receptor expressed by all TAP$^-$ NK cells, but by no more than a few TAP$^+$ NK cells (Vitale et al., 2002). Support for this hypothesis was provided by the demonstration that CEA-CAM1 is expressed by most chronically activated TAP$^-$ NK cells and inhibits the lysis of autologous PHA T-cell blasts through homotypic interaction (Markel et al., 2004b). However, TAP$^-$ CEA-CAM1-negative T cells were also unable to kill autologous T-cell blasts, which indicates that other still undiscovered mechanisms are involved in the tolerance of activated NK cells to autologous T-cell blasts in these patients (Markel et al., 2004b, and unpublished observations).

Autoreactive $\gamma\delta$ T cells have also been described in TAP-deficient patients. Interestingly, biopsies of skin lesions revealed the presence of foci of activated NK cells and $\gamma\delta$ T cells in the dermis, suggesting a role of these cells in the development of skin lesions (Moins-Teisserenc et al., 1999).

TAP-independent anti-EBV $\alpha\beta$ T-cell responses have been demonstrated in two cases of TAP deficiency. In one study, a cytotoxic CD8$^+$ $\alpha\beta$ T-cell clone was characterized that recognized a peptide of latent membrane protein 2 (LMP2) presented by HLA-B molecules (de la Salle et al., 1997). The LMP2 antigen was also found to be presented by HLA-A2 on TAP-deficient cell lines (Lee et al., 1996). In other work, an anti-EBV T-cell clone recognizing the EBV transcription factor BMFR1 presented by HLA-B7 was isolated from another TAP-deficient patient (de la Salle et al., 2002). Therefore, CD8$^+$ $\alpha\beta$ T cells can contribute to immune responses against viruses in TAP-deficient patients through recognition of TAP-independent antigens presented by classical HLA class I molecules.

A MODEL FOR THE PATHOLOGY

At first glance, the absence of susceptibility to viruses and the predominance of pulmonary bacterial infections in TAP-deficient patients may appear surprising. Our analysis nevertheless demonstrates that cytotoxic CD8$^+$ $\alpha\beta$ T cells recognizing TAP-independent viral antigens can be stimulated in vivo and suggests that activated NK cells may also play a role in immune defense. These cell-mediated cytotoxic responses are less efficient than in normal individuals because (1) TAP-independent antigens are rarer and (2) activated TAP$^-$ NK cells are less cytotoxic than those from normal subjects. Although a higher production of antibodies may partly compensate for these weaker cell-mediated responses, the overall immune response is unlikely to be sufficient. Consequently, TAP deficiency should lead to a delayed clearance of viral infections, as observed in β2m-deficient animals (Raulet, 1994).

Several viruses, including respiratory syncytial virus, are known to induce synthesis of IL-8, a chemoattractant for neutrophils and for a subpopulation of T lymphocytes. Since the lungs are probably more subject to viral infections than other tissues, these organs would be attacked (1) by the viruses,

which are inefficiently cleared, and (2) by proteolytic enzymes released from neutrophils present in large numbers due to the sustained production of IL-8. In addition, the presence of MCP1 and IL-2 have been documented in the bronchoalveolar fluids of TAP-deficient patients with chronic inflammation (Hanna et al., 2005). Whether activated NK cells, and also cytotoxic $CD8^+$ αβ or γδ T cells, further kill uninfected HLA class I-deficient autologous lung cells, if no inhibiting receptors are engaged, remains to be clarified. On the other hand, expression of CEA-CAM1 counter-receptor, CEA1, on epithelial and endothelial cells (Obrink, 1997) might provide protection against activated CEA-CAM1$^+$ NK cells. If this pathway does exist, it could be antagonized by bacteria such as *H. influenzae*, which express CEA-CAM1 ligands (Virji, 2001). It should be noted that the concentration of soluble CEA-CAM1 in the blood of TAP-deficient patients is abnormally low (Markel et al., 2004a), which suggests that it is consumed in these chronically infected individuals.

Poor control of inflammatory cytotoxic responses may lead to destruction of ciliary cells and fibrosis of the lungs, with the result that bacteria and/or bacterial endotoxins are inefficiently eliminated, thereby increasing the chemotaxis of neutrophils and maintaining a state of chronic inflammation in the lungs. It has been shown that cystic fibrosis patients display inflammation of the lungs with an associated increased neutrophil count within the first weeks of life, several years before the appearance of pulmonary insufficiency (Khan et al., 1995). Similarly, the progressive degradation of lung tissues in TAP-deficient patients is probably mediated by neutrophils and may begin long before any observable pathology. As mentioned above, chronic bacterial colonization of the lungs can lead to the development of ANCA, which may exacerbate the pathology (Schultz, 2007).

One poorly understood aspect of the pathology is the development of skin lesions at any age and only in some patients. No pathogens could be detected in these lesions (Gadola et al., 2000). The activated NK and γδ T cells present in inflamed tissues, which kill autologous cells in TAP-deficient patients, may be involved, and histological studies support this hypothesis (Moins-Teisserenc et al., 1999).

STRATEGIES FOR DIAGNOSIS

Until recently, HLA class I deficiency was diagnosed by serological HLA typing. This technique is now rarely used and has been replaced by molecular typing, which, in this case, is partially informative. Thus, if molecular typing shows that the patient is HLA homozygous, this will be a strong indication that the defect is linked to HLA and might therefore be a tapasin or TAP deficiency. HLA typing is particularly relevant in the case of consanguineous families. However, although the situation has not yet been encountered, an absence of HLA homozygosity does not formally eliminate such a deficiency. In fact, an HLA homozygous TAP deficiency in a context of unrelated parents has been described (Parissiadis et al., 2005).

Flow cytometric analysis of PBMCs labeled with the anti-class I monomorphic monoclonal antibody W6/32 is a rapid and convenient way to confirm a suspicion of HLA class I deficiency. This test should also be carried out on PBMCs from other members of the family (parents and first-degree relatives). In this context, infection of a cell line derived from the patient with a recombinant vaccinia virus expressing TAP1, TAP2, or both subunits (Russ et al., 1995), followed by flow cytometry of cells stained with W6/32, will determine whether the deficiency results from a TAP defect and if so, which TAP gene is mutated. Somatic complementation, by fusing EBV B cells or T-cell blasts, from the patient with control $TAP1^-$- and $TAP2^-$-deficient lymphoblastoid cell lines also allows reliable identification of the deficiency (Markel et al., 2004b). The defect will then be characterized by RT-PCR analysis, followed by direct sequencing.

TREATMENT AND PROGNOSIS

Bronchiectasis and bacterial colonization of the respiratory tract lead to chronic respiratory failure and a profound reduction in life expectancy. As in cystic fibrosis and other inherited dysfunctions of the respiratory mucosa, the evolution of bronchiectasis is closely linked to the bacterial charge. Since no specific therapy can yet be proposed for HLA class I-deficient patients, the only way to limit the inflammatory process and hence the neutrophil-mediated lung damage is to aggressively treat and, if possible, prevent the episodes of bronchial infection. When the diagnosis is made in infancy, for instance, in the case of early detection in the family of a known patient, nurseries and other infant communities should be avoided to reduce the risk of early viral contamination. Immunizations against measles, pertussis, and influenza must be carefully controlled, and exposure to tobacco smoke in the home environment should be prohibited. Bacteriological examination of the sputum is mandatory to provide guidelines for antimicrobial therapy. *H. influenzae* and *S. pneumoniae* strains, which are commonly found in repeated sputum tests from such patients, can be treated with oral antibiotics (β-lactams with penicillinase inhibitors, macrolides), while intravenous treatment is advised in the event of a major bacterial inoculum (bronchorrhea). Some other types of gram-negative bacilli such as enterobacteria and nonmucoid *P. aeruginosa* strains may also be found in sputum and require intravenous administration of third-generation β-cephalosporins and aminoglycosides. Low doses of erythromycin were reported to improve and stabilize chronic inflammation of the respiratory tract in a patient treated for 19 years (Azuma et al., 2001; Furukawa et al., 2000). This antibiotic is also an inhibitor of neutrophil functions and is used in the treatment of diffuse panbronchiolitis in Far Eastern countries, which supports the hypothesis that the lung disease of TAP-deficient patients is mediated by neutrophils.

A long-term policy for antimicrobial therapy is difficult to define, as the bronchial colonization does not immediately endanger the survival of these HLA class I-deficient individuals. However, continuous or alternate use of oral antibiotics becomes necessary when bronchiectasis develops. Regular intravenous administration of high-dose antibiotics increases the patients' level of comfort and probably slows their progressive functional impairment.

Pulmonary function tests, which should be performed at regular intervals, will initially show bronchial obstruction unresponsive to inhaled bronchodilators. Inhaled corticosteroids have been tried at this stage, but without clear clinical benefit. Overdistention of the chest and chronic respiratory failure occur secondarily and lead to end-stage disease.

CT scans and 99m-Technetium pulmonary scintigraphies are useful tools for assessment of bronchiectasis. If localized lesions are suspected, preoperative bronchography and surgical lobectomy should be considered, although to our knowledge no HLA class I-deficient patient has to date undergone thoracic surgery. Since the mucosal injuries of type I BLS also involve the upper airways, nasal endoscopy and sinus CT scans can be proposed in the case of suspected sinusitis with nasosinusal polyps. The treatment of such lesions is surgical (polypectomy, ethmoidectomy) or medical (local washing, high-dose topical corticosteroids), or requires both types of intervention. Surgical treatment of chronic sinusitis was reported to accelerate the nasal disease in one case (Gadola et al., 2000) but not in another (Azuma et al., 2001). Chronic otitis media must be carefully checked on regular audiograms and tympanograms. Surgical operations should be performed only under high doses of intravenous antibiotics, and placement of tympanostomy tubes necessitates prolonged antibiotic therapy to avoid middle ear suppuration.

The skin ulcers and granulomas are difficult to cure and have dramatic, even tragic incidences on the life comfort and the sociability, especially in the cases of facial injuries. Trials with IFN-α or IFN-γ made the lesions worse, while skin grafting was followed by the reappearance of lesions at the same sites (Willemsen et al., 1995). Only basic antiseptic care can be recommended (Gadola et al., 2000), but relapse occurs fairly regularly.

One unexplained question is why some patients are moderately affected whereas others are severely ill. In two different families living in nearby areas, the patients had the same homozygous HLA haplotype (de la Salle et al., 2002; Markel et al., 2004b) and the same mutation (O. Mandelboim, personal communication). Nevertheless, in one family the two TAP-deficient adults had an excellent health status, while in the other family four children experienced severe lung lesions. Hence, environmental factors or polygenic traits must influence the clinical course of these patients.

Concerning the two patients followed in Strasbourg, their deficiency was diagnosed when they were 8 and 12. At this time the bacterial charge was of major concern for the eldest, requiring continuous oral antibiotics and intravenous treatment during infectious exacerbations. This policy did not induce any bacterial resistance and stabilized the bronchiectasis. The bronchorrhea gradually disappeared and antibiotics were withdrawn at the age of 20 with no further functional impairment, as is the usual pattern in bronchiectasis caused by non-cystic fibrosis. The younger patient has experienced a similar clinical course.

Bone marrow graft was attempted once in a patient with chronic skin ulcers, followed by clinical improvements (Khakoo et al., 2008). Nevertheless, since then, no definitive report has been published, questioning whether this therapy is appropriate.

ANIMAL MODELS

MHC class I-deficient transgenic mice have been generated by disrupting the genes encoding β2m (Koller et al., 1990; Zijlstra et al., 1990), the TAP1 subunit (Van Kaer et al., 1992), or tapasin (Grandea et al., 2000). The findings obtained with the first two models are discussed in detail in three reviews (Hoglund et al., 1997; Ljunggren et al., 1996; Raulet, 1994). These two deficient strains express low levels of MHC class I molecules on the plasma membranes and have very low absolute numbers of $CD8^+$ αβ T cells. In β2m-deficient mice, $CD8^+$ αβ T cells can reject allogeneic tumor cells and syngeneic tumor cells expressing minor antigens (Apasov and Sitkovsky, 1994). The mice can fight viral infections, but with lower efficiency than normal animals. Thus, attenuated influenza viruses are cleared more slowly and virulent strains inflict a higher rate of mortality, while β2m-deficient mice cannot eliminate Theiler's virus, although they survive early infection. The mechanisms of resistance to these and other viruses, such as Sendai virus, rotavirus, reovirus, and herpes simplex virus, appear to involve $CD4^+$ T cells and antibody production. The development of $CD8^+$ αβ T in TAP-deficient mice is impaired, but not as severely as in β2m-deficient mice (Ljunggren et al., 1996). In these animals, HSV glycoprotein B cytotoxic T cells specific to TAP-independent antigens were induced by immunization with a recombinant vaccinia virus or naked DNA, but not following HSV infection (Paliard et al., 2001). TAP-deficient animals were more susceptible to infection by the intracellular pathogen *Mycobacterium tuberculosis* (Behar et al., 1999), whereas they were not particularly susceptible to tumor formation (Johnsen et al., 2001).

The NK cells of β2m-deficient mice displayed reduced cytotoxicity but could be activated in vivo by viral infection (Su et al., 1994; Tay et al., 1995). The NK cells of these animals, like those of β2m-deficient mice, displayed reduced activity (Ljunggren et al., 1994). A study of MHC class I-deficient animals strongly suggested that their resting NK cells are self-tolerant due to reduced stimulation of inhibitory receptors (Joncker and Raulet, 2008).

FUTURE DIRECTIONS

Most known HLA class I deficiencies result from a defect in one of the two TAP subunits. HLA class I deficiency should be systematically investigated in cases of unexplained chronic inflammation of the respiratory tract leading to the development of nasal polyps, granulomatous lesions of the midface or legs, or bronchiectasis or chronic bacterial infection of the lungs, notably with *H. influenzae*. On the other hand, the fact that TAP-deficient adults can be healthy for decades indicates that TAP deficiency could come to the fore in atypical situations. It should be emphasized that in the course of our laboratory work, the first profiles of TAP-deficient patients (chronic inflammation of the respiratory tract, absence of phenotype, ocular toxoplasmosis) were identified after HLA typing had been requested on the basis of an inappropriate rationale. In the absence of clinical data on "conditional" HLA class I

deficiencies, the discovery of such defects is at present incidental. It will be necessary to collect data on the health status of these individuals and to elucidate the genetic defect(s) responsible for their deficiency.

The process of lung tissue degradation in HLA class I deficiency is likewise not yet fully understood, and efficient methods of treating the lung disease need to be developed, for example by testing drugs modulating neutrophil activity. The etiology of the skin lesions also requires investigation. The apparent absence of pathogens in the lesions needs to be reassessed, since over the past few years, thanks to the development of high-throughput sequencing techniques, several "new" viruses have been discovered. Effective care of the skin lesions remains a fundamental problem. Progress may result from the discovery of drugs targeting granuloma formation or survival. Interestingly, the lesions of one patient transiently regressed when she was pregnant (Furukawa et al., 2000), as has often been observed for other clinical manifestations of autoimmune diseases. This regression suggests that favorable anti-inflammatory responses are induced during pregnancy. Finally, the influence of HLA class I deficiency on the production of antibacterial antibodies requires clarification.

At a more fundamental level, as suggested by our own studies of anti-EBV T-cell responses in TAP-deficient patients, analysis of the antiviral responses mediated by $CD8^+$ $\alpha\beta$ T cells in these individuals might lead to the identification of a large number of viral proteins presented in a TAP-independent manner. Such observations could provide insight into the molecular bases and physiological relevance of these TAP-independent presentation pathways.

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18.

RETICULAR DYSGENESIS

Wilhelm Friedrich, Manfred Hoenig, Ulrich Pannicke, and Klaus Schwarz

HISTORY

Severe combined immunodeficiency (SCID) in association with congenital aleukocytosis was initially reported in 1959 by De Vaal and Seynhaeve in monozygotic prematurely born male twins who were noted at birth to completely lack white blood cells (WBC) (de Vaal and Seynhaeve, 1959). Histomorphologic findings in these infants, who both died as neonates from septic complications, revealed abnormalities consistent with SCID, with lack of lymphoid cells in lymphoid organs and with thymic dysplasia. In addition, bone marrow contained no myeloid cells and no lymphoid cells, while erythroid development was normal and megakaryocytes were present. The authors chose the term "reticular dysgenesis" (RD) based on the assumption that the disorder resulted from a defect of primitive multipotent "reticular" cells selectively failing to develop into myeloid and lymphoid precursor cells. Subsequently other cases with similar findings were reported, except that in some, WBCs were not completely absent and in the marrow, immature myeloid cells up to the promyelocyte stage were present (Alonso et al., 1972; Espanol et al., 1997; Gitlin et al., 1964; Haas et al., 1977; Ownby et al., 1976). An informative case study was published by Roper et al. using extensive cell-surface marker staining of marrow cells to dissect the various maturation stages (Roper et al., 1985). The authors found cells of all maturational stages of both lymphoid and myeloid development in the marrow, with a predominance of immature cells and almost complete depletion of myeloid cells beyond the promyelocyte stage, respectively of B cells beyond the pre-B-cell stage. Interestingly, also a substantial proportion of mature OKT3-positive T cells were observed in the marrow. These findings suggested to the authors that the defect in RD is an undefined abnormality interfering with late lymphoid and myeloid cell differentiation rather than an early stem-cell defect as proposed in the original description of the disorder. In 1983 the first successful treatment of a patient with RD by bone marrow transplantation (BMT) was reported, providing evidence that the disease is manifested within the stem cells and its progeny rather than in bone marrow microenvironmental elements (Levinsky and Tiedeman, 1983). It was also observed that in contrast to patients with other forms of inherited congenital neutropenias, treatment with G-CSF in RD was without any effect on granulopoiesis (Bujan et al., 1993). More recently, when more patients became evaluable after successful transplantation, primary sensorineural deafness was recognized as another hallmark of the disorder (Small et al., 1999). Very recently two groups independently described the molecular basis of the disorder to be a deficiency of adenylate kinase 2 (Lagresle-Peyrou et al., 2009; Pannicke et al., 2009).

CLINICAL AND LABORATORY PRESENTATION

There are a number of unusual clinical aspects that set patients with RD apart from patients with other forms of SCID (Table 18.1). Almost without exception patients come to medical attention during the neonatal period because of suspected neonatal infection and sepsis, findings of immaturity, or a combination of both. There is a high incidence of low birth weight, commonly due to both prematurity and intrauterine growth retardation, as based on a review of available data in 16 RD patients. Birth weights were below 2,500 g in 13 cases and even below 1,500 g in 5 cases. This is rather distinct from patients with other SCID variants, who are usually delivered at term and appear healthy without evidence of intrauterine growth retardation. Significant hepatomegaly

Table 18.1 **CLINICAL AND LABORATORY ABNORMALITIES IN RETICULAR DYSGENESIS DISTINCT FROM OTHER VARIANTS OF SEVERE COMBINED IMMUNODEFICIENCY**

- Low birth weight secondary to prematurity and/or intrauterine growth retardation
- Infectious complication during the neonatal period with high fatality rate
- Profound blood leukopenia and agranulocytosis
- Red cells normal/decreased
- Thrombocytes normal/decreased
- Marrow cellularity normal/decreased
- Maturation arrest of myeloid cells in bone marrow

and/or splenomegaly was noted in three cases, due in one case to cholestatic liver disease, in the others of unknown etiology. Abdominal symptoms secondary to intestinal stenosis were observed in two other cases requiring surgical intervention. In all patients surviving after transplantation, bilateral sensorineural deafness became evident (Small et al., 1999). Hearing assessment by brain stem-evoked response audiometry, applicable already in the small infant, has been useful to confirm the diagnosis of RD (Reubsaet et al., 2007).

Profound leukocytopenia and agranulocytosis represent the leading laboratory findings in RD. Automated WBC counting occasionally indicates a falsely higher leukocyte count when substantial numbers of nucleated red cell precursors are transiently present in the circulation. An analysis of hematological findings in 14 recently reported cases where the diagnosis of RD was confirmed by molecular analysis defines the range of hematological abnormalities in RD. WBC numbers have ranged from less than 100/uL to 1,600/uL (mean 630/uL). Granulocytes most commonly were completely absent, only exceptionally reaching at maximum 150/uL; monocytes were usually detected, mostly in lower-than-normal numbers. Of note, eight patients had neonatal anemia, with hemoglobin at birth as low as 3.7 g/dL and 5.4 g/dL, and in the others between 8 and 12 g/dL (normal 13.5 to 19 g/dL). Five patients showed marked thrombocytopenia, with platelet numbers below 50,000/uL in three cases in association with marked anemia. There was no apparent correlation of these hematological deficiencies with septic complications. Characterization of lymphocytes by surface marker analysis can reveal the presence of low numbers of CD3-positive T cells. In one study, six of six analyzed patients with RD were found to have very low numbers of T cells; in each case, these cells were noted to originate from an intrauterine maternofetal transfusion (Müller et al., 2001). This phenomenon of maternal T-cell engraftment is also found in other SCID patients but appears to be rather common in RD. In three of the six patients with maternal T cells, this engraftment was clinically inapparent, while three patients had manifestations of histologically proven graft-versus-host disease, involving in two cases the skin with mild transitory rashes and in one the liver with severe cholestatic liver disease at birth.

Examination of bone marrow aspirates and biopsies is helpful to confirm the diagnosis of RD; however, obtaining representative marrow samples in newborns, in particular in premature ones, may be difficult technically, and as a consequence adequate morphological assessment not always feasible. If available, the most prominent abnormality is the complete absence of mature myeloid cells. The marrow usually is normocellular and is dominated by immature myeloid cells up to the promyelocyte stage, with normally developing erythroid cells and with the presence of megakaryocytes. A representative marrow morphology is depicted in Figure 18.1.

MOLECULAR BIOLOGY OF RD

The defective gene in reticular dysgenesis, *adenylate kinase 2 (AK2)*, was discovered by homozygosity mapping and candidate gene sequencing (Lagresle-Peyrou et al., 2009; Pannicke et al., 2009).

The phylogenetically highly conserved *AK2* gene is located on chromosome 1p35.1 and encompasses about 24 kb. Two alternatively spliced mRNAs (isoform A and isoform B) are ubiquitously expressed (except for erythrocytes), albeit differing in relative expression. Surprisingly, AK2 protein was detected in the lumen of the capillaries and terminal blood vessels of the cochlear stria vascularis region in postnatal day 7 mice but not at birth, suggesting that AK2 may function as an ecto-enzyme (Lagresle-Peyrou et al., 2009). Isoform A encodes a protein of 239 amino acid length, while isoform B encodes 232 amino acids. Both isoforms differ only in a few residues at the C-terminal end.

AK2 is a nucleus encoded mitochondrial protein and is located in the intermembrane space of mitochondria. AK2 catalyses the reaction AMP + ATP $\leftrightarrow$ 2ADP. The ADP is transported into the mitochondrial matrix and serves as the phosphate acceptor of complex V of the electron transport chain during oxidative phosphorylation.

AK2 has an ATP and NMP binding domain as well as a LID domain. The extensive movement of its domains during catalysis has made AK2 a model for molecular dynamic studies (Schlauderer et al., 1996).

In addition, AK2 has been implicated in a novel apoptotic pathway through formation of a complex with FADD and caspase-10 (Lee et al., 2007).

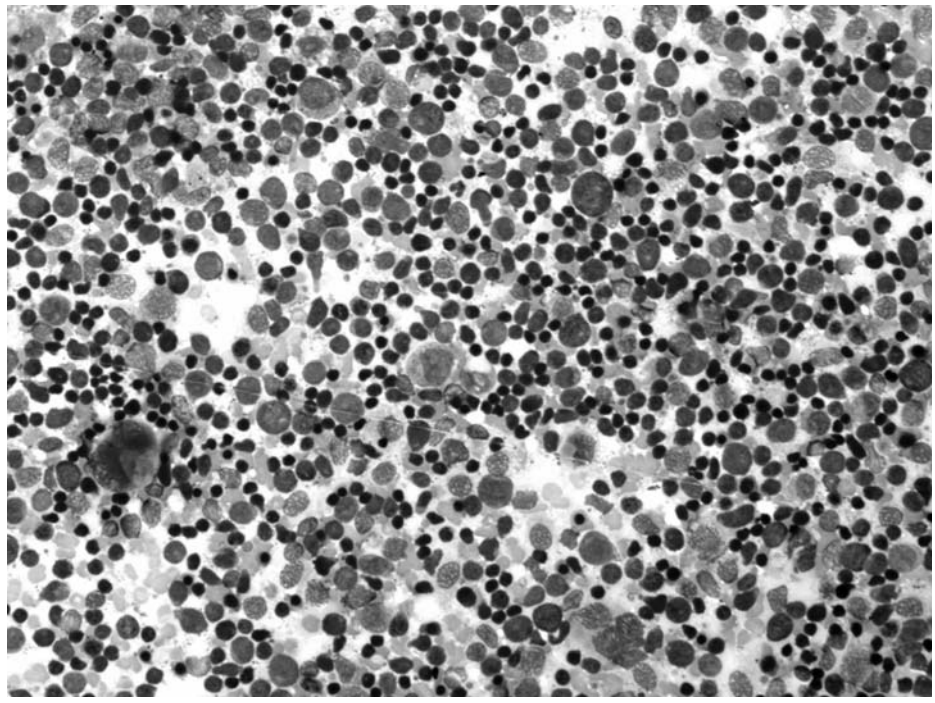

Figure 18.1 Representative bone marrow aspirate of a patient with reticular dysgenesis (May Grunwald/Giemsa staining, Olympus, ×1,000). (See Color Plate.)

MUTATION ANALYSIS OF RD PATIENTS

Pedigree analyses have confirmed that AK2 deficiency is an autosomal recessive genetic syndrome and affected children have failed to inherit normal alleles of the *AK2* gene. The genetic alterations are summarized in Table 18.2. The mutations are distributed over the complete gene. The spectrum of mutations includes small and large deletions, splice-site mutations, and missense as well as nonsense mutations. The majority of mutations are homozygously inherited in accordance with the consanguinity of the families. Each family bears its private mutation(s), excluding a founder effect. The mutations did not show any influence on AK2 steady-state RNA levels (Lagresle-Peyrou et al., 2009; Pannicke et al., 2009).

When tested, all of the identified mutations severely decrease the stability of the protein. The missense mutant p.Asp165Gly is expressed only in low levels (~10 percent) in an EBV line but not in fibroblasts of the patient, the missense mutant p.Arg103Trp shows residual expression (~10 percent) in fibroblasts, and all other mutants are null mutations.

One individual with all the clinical and cellular characteristics of RD exhibited no *AK2* mutations, suggesting that RD may be caused by genocopies (Pannicke et al., 2009).

The molecular pathophysiology of the AK2 deficiency is not clear at present either for the loss of leukocytes or for the sensorineuronal deafness. Nontransformed primary dermal fibroblasts of patients exhibit a higher propensity for apoptosis with increased reactive-oxygen levels and a lower mitochondrial membrane potential (Pannicke et al., 2009).

ANIMAL MODELS OF AK2 DEFICIENCY

In zebrafish, one of the human mutations (F3, Table 18.2) was mimicked by designing a splice-site morpholino. This led to aberrant splicing of the zebrafish *ak2* gene predicted to result in the translation of a nonfunctional protein. Interference with Ak2 function did not affect the overall development of embryos; likewise, neither immature hematopoietic cells nor erythropoiesis, as evidenced by the presence of circulating red blood cells, were affected in morphants. In contrast, developing lymphocytes (best revealed by the appearance of T cells in the thymus anlage) were absent in the Ak2 morphants. This indicates that the phenotype has a high degree of specificity and that Ak2 has an evolutionarily conserved function in leukocyte development (Pannicke et al., 2009).

The effects of an *Ak2* gene knockout on the phenotype of Ak2 mutants were examined using P-element technology in drosophila (Fujisawa et al., 2009). Although homozygous Ak2 mutated embryos developed without any visible defects, their growth ceased and they died before reaching the third instar larval stage. Maternally provided AK2 mRNA was detected in fertilized eggs, and weak AK2 activity was observed in first and second instar larvae of the homozygous AK2 mutants, suggesting that maternally provided AK2 is sufficient for embryonic development. Disappearance of AK2 activity during larval stages resulted in growth arrest and eventual death. Final conclusions regarding the distinct phenotypes of the diverse animal models and humans awaits more sophisticated modeling in mice, most using conditional alleles.

Table 18.2 AK2 MUTATIONS IN INDIVIDUALS WITH RETICULAR DYSGENESIS

FAMILY (NUMBER AFFECTED)	ALLELES	MUTATION	PREDICTED EFFECT	DETECTABLE PROTEIN IN FIBROBLASTS
F1	homozygous	c.636_*2601del	p.Ser213AspfsX21	no
F2	compound heterozygous	c.118delT c.1A>G	p.Cys40ValfsX5 p.Met1Val	no
F3	homozygous	c.331–1G>A	aberrant splicing	no
F4 (2)	homozygous	c.453delC	p.Tyr152ThrfsX12	no
F5	homozygous	c.498 + 1G>A	aberrant splicing	no
F6 (2)	homozygous	c.494A>G	p.Asp165Gly	no; EBV cells low
F7	homozygous	c.546delC	p.Leu183X	no
F8	compound heterozygous	c.556C>T c.94_219del126	p.Arg186Cys p.Ala32_Leu73del	n.a.
F9	homozygous	c.307C>T	p.Arg103Trp	low
F10	homozygous	c.633del5kb	p.Lys233X	no
F11	homozygous	c.25G>T	p.Glu9X	n.a.
F12	compound heterozygous	c.400–401delCT c. 614–615delGG	p.Leu134AlafsX32 p.Gly205AspfsX28 (isoA) p.Gly205AspfsX92 (isoB)	n.a.

Mutations were accumulated from Lagresle-Peyrou et al., 2009; Pannicke et al., 2009; and Poliani et al., 2009.

Mutations were denoted according to the recommendations for the description of sequence variations (version February 2008) of the Human Genome Variation Society (Den Dunnen et al., 2000).

n.a., not available.

TREATMENT

Since the first report in 1983 of successful treatment of RD by hematopoietic stem cell transplantation (HSCT) (Levinsky and Tiedman, 1983), this therapy has been explored in a number of patients (Antoine et al., 2003; Bertrand et al., 2002; de Santes et al., 1996; Friedrich et al., 1985; Heltzer et al., 2007; Reubsaet et al., 2007). Because of the rare availability of HLA-identical siblings as donors, most patients were transplanted from HLA-haploidentical family donors or from HLA-matched unrelated donors. In the initial report, the donor was an HLA-identical sibling and the patient had received no preparative conditioning prior to transplantation. After transplantation significant graft-versus-host disease developed, complicated by marrow failure, but subsequently normal hematological and immunological functions developed, with evidence that all blood cells were of donor origin. Contrasting to this experience, it became evident that patients with RD undergoing transplantation in the absence of myeloablative conditioning are prone to fail reconstitution of myeloid functions, with persistence of agranulocytosis, presumably because colonization by donor stem cells requires prior depletion of stem-cell niches in the marrow. Although the need for myeloablative conditioning adds to its risks, HSCT needs to be implemented in RD patients as soon as possible because of the extremely high risk of the rapid development of fatal infectious complications. Meanwhile, a number of patients are long-term survivors, clearly attesting to the potential of this therapy to permanently reconstitute a normal blood cell and immune system and to cure the disorder (Antoine et al., 2003; Heltzer et al., 2007).

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19.

CD8 DEFICIENCY

Teresa Español and Esther Mancebo

CD8 serves as a co-receptor for T-cell receptor (TCR) recognition of MHC class I-associated peptides (Gao et al., 2000; Zamoyska et al., 1998), which in turn leads to cytotoxic αβ T lymphocyte (CTL) activation and lysis of the target cell. This mechanism enables CTLs to recognize and eliminate infected cells, tumor cells, and allogeneic cells. TCR complex signaling activates CD8 adhesion molecule function, resulting in a CD8 interaction with MHC-I that is sufficient to maintain firm T-cell adhesion (Varghese et al., 2008). The kinetics of CD8 and MHC interaction at the cell membrane has been measured (Huang et al., 2007), and crystal structure studies have defined the binding mode of CD8αβ to MHC-I (Shore et al., 2008).

CD8 molecules are expressed on the cell surface as either αα homodimers or αβ heterodimers. Surface expression of CD8β is dependent on expression of CD8α, as CD8β polypeptides in the absence of CD8α are retained in the endoplasmic reticulum and degraded. Both chains (α and β) are composed of a single extracellular immunoglobulin-like domain, a membrane-proximal hinge region, a transmembrane domain, and a cytoplasmic tail. CD8 associates with β_2m and the α2 and α3 domains of MHC class Ia molecules. This association increases the adhesion/avidity of the TCR to its class I target, and in addition, CD8 associates with the *scr* tyrosine protein kinase $p56^{lck}$, leading to rapid activation of the cytotoxic T lymphocyte via internal signaling events (Chapupny et al., 1998). Expression of CD8 is characteristic of CTLs and is critical for their progression through the process of positive selection during differentiation in the thymus (Zamoyska et al., 1998).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Only two unrelated familial cases with CD8 deficiency are described in the literature; both families are consanguineous and of Spanish Gypsy origin. The main clinical manifestations are recurrent infections of the respiratory tract. The first case reported (de la Calle et al., 2001) was a 25-year-old man admitted with respiratory distress, weight loss, and general malaise. He had suffered repeated bouts of bronchitis and otitis media from childhood. Chest X-ray and CT scan revealed disseminated bronchiectases. Sputum culture was positive for *Haemophilus influenzae*. Functional respiratory tests revealed severe mixed ventilatory disturbance. His clinical status improved after intravenous antibiotic therapy. He required further admissions because of respiratory reinfections of his extensive bronchiectases (*Pseudomonas aeruginosa* was detected in sputum on several occasions). By age 33, his ventilatory function had deteriorated to the point where he became wheelchair-bound and dependent on oxygen. The patient died before being able to undergo lung transplantation. This patient was the fourth of nine siblings of first-cousin parents. The remaining family members, including two sisters with CD8 deficiency (Fig. 19.1), do not have clinical disease.

The second case reported (Mancebo et al., 2008) was an 18-year-old woman, the first of three siblings of second-cousin parents. She suffered from recurrent respiratory infections starting at 1 month of age, including bronchiolitis, pneumonia, and otitis. On separate occasions, serologies were positive for RSV and parainfluenza 3 viruses. She required repeated hospitalizations. At the age of 4 years, she was diagnosed with asthma, otitis, sinusitis, and lung atelectasis. At 7 years

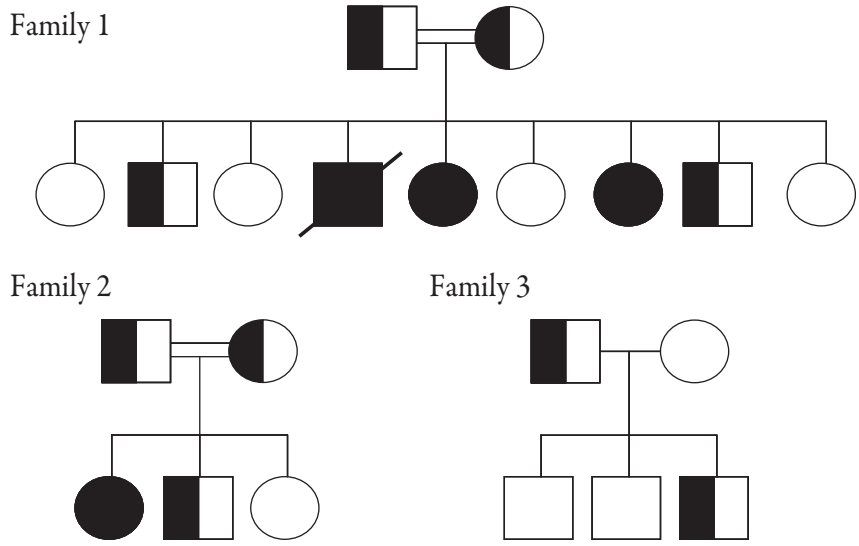

Figure 19.1 Pedigrees of families 1, 2, and 3.

of age, the patient was referred to an endocrinologist for failure to thrive. She had received all routine vaccinations, tests for allergy were negative, and serum immunoglobulin levels were normal. Although her infections resolved with antibiotic treatment, at 15 years of age she suffered two episodes of pneumonia together with several respiratory infections, with high temperature and productive cough.

LABORATORY FINDINGS

The main laboratory findings are shown in Table 19.1.

Total IgG and IgG subclasses were normal. Serologies were positive for tetanus, toxoplasma, *Mycoplasma pneumoniae*, CMV, herpes zoster, herpes simplex, and rubella in patient 1 and for CMV, EBV, and rubella in patient 2. Serologies were negative for HIV, EBV, *Legionella pneumophila*, aspergillus, and brucella in patient 1 and for HIV, hepatitis (A, B and C), syphilis, and toxoplasma in patient 2. Autoantibodies were negative in both patients.

Lymphocyte proliferation and subpopulations were normal in both patients, except that CD8 surface expression was not detected on either $CD3^+$ or $CD3^-$ cells (Table 19.1). Double-negative (DN) T cells ($TCR\alpha\beta$ + $CD4^-$ $CD8^-$) were increased (16 percent in patient 1 and 10 percent in patient 2; reference range = 0.1 to 2 percent). The predominant immunophenotype found in these α/β DN T cells of patient 1 was $CD3^{hi}$ $CD5^+$ $CD2^+$ $CD45RA^+$ $CD57^+$ $CD11b^+$ $CD28^-$, with a large Vβ repertoire. A more extended study in patient 2 and in a CD8-deficient sister of patient 1 revealed that DN T cells exhibit mostly an effector-memory or effector phenotype, respectively, whereas naïve T cells are underrepresented. Although, DN T cells from both patients show low TRECs levels and a skewed Vβ repertoire (Bernardo et al., 2011).

Upon stimulation, the DN T cells of the sister of patient 1 were positive for intracellular interferon-γ (IFN-γ), but not for interleukin (IL)-2 or IL-4. The percentage of TCR γδ T cells was normal, and weak CD8β expression was detected by Western blot in peripheral blood mononuclear cells (PBMCs); however, CD8β membrane expression was absent.

FAMILY STUDIES

Whole blood samples from the patients and their relatives were stained with an extensive panel of anti-CD8α monoclonal antibodies. Two asymptomatic younger sisters of patient 1 also lacked surface $CD8^+$ (α or β) expression and showed weak intracellular CD8β and high numbers of DN αβ T cells (10 percent and 4 percent, respectively). Mean fluorescence intensity of CD8α expression on cell surface and soluble CD8 (by ELISA test) were decreased in the parents and two brothers (II-2 and II-8) of patient 1 and in the parents and one brother (II-2) of patient 2 (all heterozygous carriers of the mutation, see below). These results indicated a good phenotype–genotype correlation.

MOLECULAR BASIS

Both patients shared a homozygous autosomal recessive missense mutation (c.331G>A) in exon 2 of the *CD8A* gene. This mutation predicts the substitution of a glycine by a serine at codon 111 (p.Gly111Ser) of the CD8α protein. Wild-type and mutated CD8, as well as chimeric CD8 molecules (CD8 MUT/WT and WT/MUT), were expressed after transfection by an expression vector (de la Calle-Martin et al., 2001), demonstrating that the presence of serine or another amino acid at position 111 precluded CD8 expression, thereby suggesting that the evolutionarily conserved glycine at this position may be important for appropriate folding of the protein. However, the lack of CD8 surface expression did not prevent development of a peripheral DN T-cell lineage with functional features resembling $CD8^+$ T cells, such as IFN-γ synthesis. Similar results have been reported in mice (Goldrath et al., 1997).

Table 19.1 Immunological Profile of CD8-Deficient Patients

	PATIENT 1	PATIENT 2
Age at diagnosis (y)	25	16
Sex	M	F
Present status	Died	Respiratory infections
Serum immunoglobulins		
IgG, M	Normal	Normal
IgA	Normal/high	Normal
IgG2	Normal	Normal
Lymphocyte subsets (cells/mm^3)		
CD3	1,080	2,185
CD4	750	1,842
CD8	0	0
TCR αβ	990	1,967
TCR γδ	90	125
CD19	105	593
CD16 + CD56	195	343
TCR Vβ usage	Normal	Normal in CD4 T cells Skewed in DN T cells
Lymphocyte proliferation		
PHA and anti-CD3	Normal	Normal
PMA + ionomycin	Normal	Normal
SEA	Normal	-
SEB	Normal	-
Tetanus toxoid	Normal	-
Allogenic cells	Normal	-
NK cell function: (K562 cell lysis)	Normal	-

FUNCTIONAL ASPECTS

CD8 deficiency is compatible with life and results in a less aggressive primary immunodeficiency than deficiency of HLA class I. We believe that the absence of classical $CD8^+$ CTL may be partially compensated for by the presence of αβ DN T cells in our patients, and by the cytolytic function of NK cells (Brown et al., 2001). In vitro, at high effector–target ratios, the cytotoxic capacity of patient DN T cells was similar to control CTLs. However, at low ratios, the cytotoxicity was significantly diminished in patients, which suggests that, in limiting conditions, cytotoxicity is less efficient in the absence of CD8 . A fraction of the $CD3^+CD4^-$ T cells from the patients were found to be positive for CMV-pentamer staining. These results show that CMV specific T cells could be generated in the absence of CD8, although in a lower grade compared with healthy individuals (Bernardo et al, 2011). Also, DN T cells present a phenotype associated with effector and memory effector cytotoxic cells. The high antibody titers to many viral infections in patients demonstrated that they had been in contact with these viruses and were immunocompetent enough to overcome these infections. Although the last infections in patient 1 were bacterial, viral infections suffered at an early age might have been responsible for the bronchial lesions that later became superinfected, as has been reported in TAP-deficient adult patients (de la Salle et al., 1999).

MUTATION ANALYSIS

Messenger RNAs for CD8α and β were detected by RT-PCR at similar levels in the CD8-negative individuals, their relatives, and normal controls. Genetic studies of CD8α were conducted in view of the above-mentioned results. For mutational analysis of patient 1, primers were chosen to generate a product covering the entire coding region and the 5′ and 3′ UTRs for CD8α by RT-PCR. PCR products were subcloned and sequenced. For patient 2, primers were designed to amplify and sequence the six *CD8A* exons surrounding genomic sequences. These analyses revealed a G-to-A transition at nucleotide 331 in exon 2 (c.331G>A) of *CD8A* in both patients. This mutation predicts the substitution of a glycine by a serine at codon 111 (p.Gly111Ser) of the CD8α protein. The glycine residue is located in the immunoglobulin domain of the CD8α chain and is conserved in all reported species through evolution, and also in the corresponding region of the closely related molecules CD8β and CD7 (Hansen et al., 2000).

Intrafamilial segregation of the mutant allele confirmed its role in the CD8 deficiency. Genetic analysis of relatives was performed by direct sequencing of genomic PCR products. In family 1, the three CD8-deficient siblings were indeed homozygous for the mutated allele ($CD8^{ser111}$). The parents and two brothers found to have low CD8 expression were heterozygous, whereas three sisters with normal CD8 levels were homozygous for the wild-type alleles (de la Calle et al., 2001). In family 2, the mutation was heterozygous in both parents and a healthy brother of the patient.

Interestingly, the only two familial cases of CD8 deficiency described have the same mutation in *CD8A* gene and are of Spanish Gypsy origin, suggesting a founder mutation. The mutation creates an additional restriction site for the enzyme *Alu*I. We took advantage of this to develop a screening test specific for the mutation, which we applied to 734 unrelated healthy subjects representing different European Gypsy populations: 242 from Spain; 394 from Bulgaria, representing 8 sub-isolates; and 98 from Romania, Hungary, and the Czech Republic. We also tested 393 unrelated Spanish non-Gypsy individuals. The c.331G>A mutation was found in heterozygosity in only 1 of the 242 Spanish Gypsy subjects, and not in non-Gypsy populations or Gypsy groups outside Spain (Mancebo et al, 2008). The three Gypsy families carrying the mutation did not share a geographical origin in Spain, and available genealogical data revealed no relationship between them; however, their common ethnicity makes it likely that a founder was the source of the mutation. Further, the three families shared a core haplotype associated with the mutation (Table 19.2).

Table 19.2 **HAPLOTYPES OF GYPSY ORIGIN ASSOCIATED WITH THE C.331G>A MUTATION IN THE *CD8A* GENE**

	FAMILY 1 AND FAMILY 2	FAMILY 3	GENOTYPE FOR CEPH INDIVIDUAL 134702	HETEROZYGOSITY (%)	ALLELE FREQUENCY IN GYPSY CONTROLS (N = 58)
D2S2232	208	210	210/218	83	208: 14%, 210: 20%
D2S388	255	255	260/264	69	255: 9%
D2S417	206	206	204/204	72	206: 21%
STSCD8A	144	144	144/160	73	144: 36%
STSCD8B	163	163	163/163	28	163: 84%
D2S2216	133	133	135/145	79	133: 4%
D2S2181	181	181	181/181	70	181: 46%

Relative order and physical distances are as follows: D2S2232-60 kb-D2S388-936 kb-D2S417-23 kb-*CD8A*:c.331G>A-3 kb-STSCD8A-40 kb-STSCD8B-1464 kb-D2S2216-81 kb-D2S2181. The marker of heterozygosity and allele frequencies were calculated from a sample group of 58 Spanish Gypsies. To allow other laboratories to compare their data with those reported in this work, we provide allele sizes for individual 134702, available from CEPH (Dib et al., 1996).

STRATEGIES FOR DIAGNOSIS

CD8 deficiency is diagnosed when either a male or female patient lacks surface expression in mature T and NK lymphocytes and has high TCRαβ + $CD4^-$ $CD8^-$ T-cell numbers and mutation in *CD8A*. The main clinical manifestations are recurrent respiratory tract infections.

CD8 deficiency should be suspected in young Spanish Gypsy patients suffering repeated and/or severe respiratory infections, and a lymphophenotype should be ordered to confirm or rule out the absence of $CD8^+$ T cells. Specific recommendations for vaccination and smoking avoidance may be proposed to affected individuals. Detection of the c.331G>A mutation by the PCR-RFLP screening test permits rapid and inexpensive diagnosis in individuals known to be at risk, permitting genetic counseling regarding this Mendelian disorder. CD8 deficiency must be differentiated from other deficiencies with low CD8 cells, such as ZAP-70 and TAP.

TREATMENT AND PROGNOSIS

Antibiotic therapy was applied according to bacterial isolates, and prophylactic antibiotics were recommended. No curative therapy (such as by bone marrow transplant) was performed in these patients. Gene therapy could, theoretically, be a therapeutic protocol. The prognosis of this immunological defect appears variable, ranging from fatal pulmonary insufficiency to mild or even asymptomatic phenotype.

CONCLUDING REMARKS

Manifestation of CD8 deficiency in humans appears less severe than in mouse models. This syndrome, like TAP1 and TAP2 deficiencies, is compatible with life and less aggressive than HLA class I deficiencies. Affected individuals have increased peripheral αβ DN T cells committed to the CTL lineage that correspond to what "should be" the $CD8^+$ population if CD8 were expressed. CD8 chains are first expressed and used by T cells early during their intrathymic development to increase the likelihood of being rescued in the event that their TCR has low affinity for MHC class I. CD8α is also expressed by human monocytes and enhances FcγR-dependent responses (Gibbings et al., 2007). However, in CD8 deficiency, T-cell clones that fulfill the functional requirements for intrathymic survival display aberrant phenotypes in periphery (DN T cells) but seem to be at least partially functional.

The diagnosis of primary immunodeficiency in adults is being increasingly reported, and it remains to be seen whether the CD8-deficient sisters of patient 1 will develop symptoms in the future. Examples of poor correlation between genotype and phenotype in other primary immunodeficiencies are described (de la Salle et al., 1994; Kornfeld et al., 1997; Sneller et al., 1997), and either modifying genes (Foster et al., 1998) or epigenetic changes (Jirtle et al., 2007) could help to explain different clinical manifestations in patients with the same genetic defect in CD8.

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20.

CRAC CHANNELOPATHIES DUE TO MUTATIONS IN ORAI1 AND STIM1

Stefan Feske

INTRODUCTION

Ca^{2+} signals contribute to the function of many immune cells, including T and B cells, natural killer (NK) cells, mast cells, dendritic cells, and macrophages. These signals control diverse functions ranging from differentiation, proliferation, gene expression, and cell motility to secretion of vesicles containing cytokines, cytotoxic, or proinflammatory proteins (Feske, 2007). The main mechanism controlling Ca^{2+} influx in lymphocytes is store-operated Ca^{2+} entry (SOCE) through the so-called calcium release activated calcium (CRAC) channel. The importance of Ca^{2+} influx through CRAC channels for immunity is highlighted by the existence of patients with a combined immunodeficiency due to a defect in SOCE, CRAC channel function, and T-cell activation (Feske et al., 1996, 2006; Le Deist et al., 1995; McCarl et al., 2009; Partiseti et al., 1994; Picard et al., 2009).

In lymphocytes, Ca^{2+} influx is initiated by engagement of immunoreceptors such as the TCR, BCR, or Fc receptors, resulting in the activation of signaling cascades (Fig. 20.1). Importantly, inositol 1,4,5-triphosphate ($InsP_3$) mediates the release of Ca^{2+} ions from the endoplasmic reticulum (ER) and activation of stromal interaction molecule (STIM) 1. Multimerization of STIM1 in the membrane of the ER leads to the opening of the store-operated CRAC channel protein ORAI1 in the plasma membrane and sustained Ca^{2+} influx from the extracellular space. The term "store-operated Ca^{2+} entry" refers to the fact that the filling state of the ER Ca^{2+} store controls the opening of calcium channels in the plasma membrane. The CRAC channel is defined by its unique functional properties, measured by patch clamping, an electrophysiological method to measure ion channel currents. Both SOCE and CRAC channels represent a universal Ca^{2+} influx mechanism employed by lymphocytes and many other cell types. Besides CRAC channels, other ion channels are indirectly involved in the regulation of Ca^{2+} influx in lymphocytes. These include the nonselective cation channel TRPM4 and the potassium channels KCNN4 and KCNA3, which together control the plasma membrane potential; a negative membrane potential is required to promote passive influx of Ca^{2+} ions through open CRAC channels and along the electrochemical gradient. Thus, theoretically mutations in a number of genes may affect Ca^{2+} levels in lymphocytes, including molecules operating proximal to $InsP_3$ production and ER store depletion as well as those affecting SOCE more directly, such as STIM1, ORAI1, potassium, and TRPM channels.

ORAI1 DEFICIENCY

BIOLOGY OF ORAI1

The CRAC channel gene *ORAI1* (or *CRACM1*, *TMEM142a*) on human chromosome 12q24 is the founding member of a new class of ion channels structurally related to *ORAI2* (*CRACM2*, *TMEM142b*) and *ORAI3* (*CRACM3*, *TMEM142c*). *ORAI1* has only two exons and encodes a highly conserved 301 amino acid protein with a tetra-spanning plasma membrane topology. ORAI1 is the pore-forming subunit of the CRAC channel. A negatively charged glutamate residue, E106, in its first transmembrane domain functions as a Ca^{2+} binding site in the ion channel pore (Prakriya et al., 2006; Vig et al., 2006a; Yeromin et al., 2006). CRAC channels are thought to be tetramers, with each ORAI1 subunit contributing a glutamate residue for coordinated Ca^{2+} binding in the CRAC channel pore. ORAI2 and ORAI3, which share the predicted tetra-spanning membrane topology with ORAI1, can form Ca^{2+} channels when ectopically expressed in vitro, and it is possible

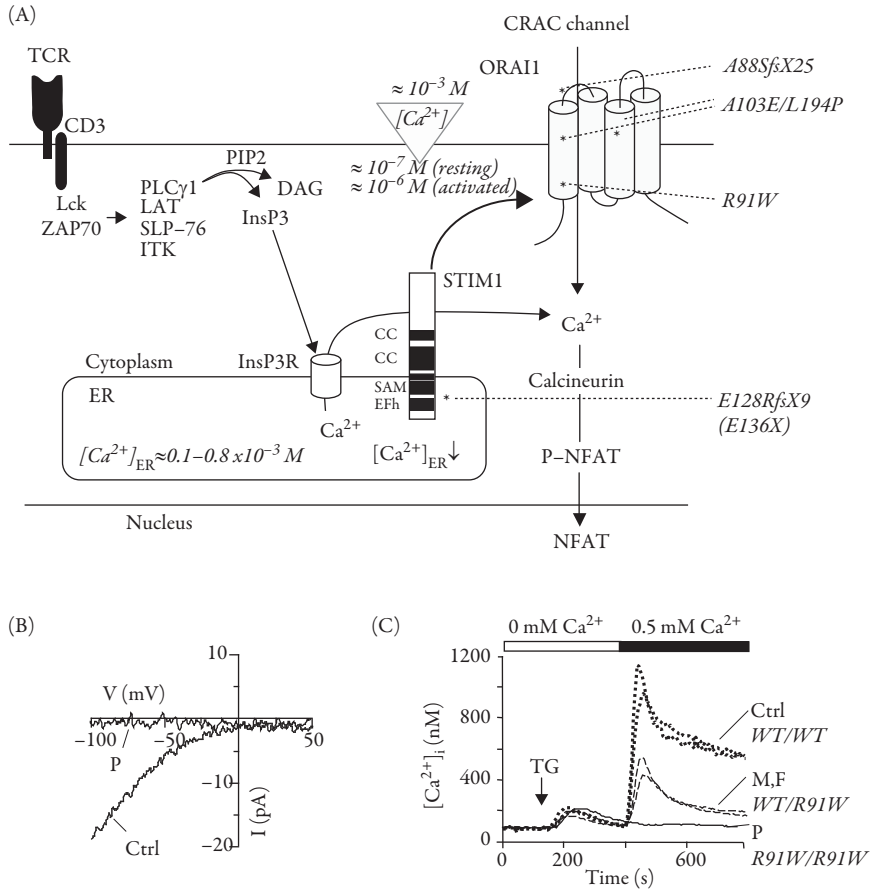

Figure 20.1 Store-operated Ca^{2+} entry (SOCE) through ORAI1 and STIM1 in T cells. (A) Following T-cell receptor (TCR) stimulation, the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ rises from ~50–100 nM at rest to ~1 μM. This increase in $[Ca^{2+}]_i$ following the activation of tyrosine kinases Lck and ZAP-70 (ζ-chain-associated protein kinase of 70 kDa), phosphorylation of adaptor proteins such as SLP76 (SH2-domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells), and activation of phospholipase (PLC) γ1. PLCγ1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) in the plasma membrane to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to and opens $InsP_3$ receptors ($InsP_3Rs$) in the endoplasmic reticulum (ER), resulting in transient efflux of Ca^{2+}. Lower ER Ca^{2+} sensed by stromal interaction molecule 1 (STIM1) leads to the opening of calcium-release activated calcium (CRAC) channels in the plasma membrane, which are composed of ORAI1 subunits. Sustained Ca^{2+} influx through CRAC channels activates calcineurin and the transcription factor nuclear factor of activated T cells (NFAT). Known deleterious mutations in *ORAI1* and *STIM1* are indicated. STIIM1 domains: EFh, EF hand; SAM, sterile alpha motif; CC, coiled-coil. (B) Absent CRAC channel current I_{CRAC} in T cells of an ORAI1 deficient patient (P, with mutation R91W) compared to those of a control (Ctrl), measured in whole-cell patch-clamp recordings (Feske et al, 2005). Cells in which ER Ca^{2+} stores had been passively depleted were subjected to voltage ramps from –100 to +50 mV. (C) Impaired SOCE in ORAI1-R91W homozygous mutant (P) vs. heterozygous parental (M, F) or control (Ctrl) T cells. Single-cell ionized calcium concentration, $[Ca^{2+}]_i$, was measured by time-lapse microscopy after T-cell loading with the Ca^{2+} indicator dye Fura-2 and stimulation with thapsigargin (TG, *arrow*) to passively deplete ER Ca^{2+} in the absence of extracellular Ca^{2+} (*open bar*). After re-addition of Ca^{2+} (*black bar*), SOCE was undetectable in patient T cells and markedly reduced in heterozygous T cells.

that endogenous ORAI2 and ORAI3 play a role in CRAC channel function and SOCE in immune cells.

MOLECULAR BASIS OF ORAI1 DEFICIENCY

Discovery of ORAI1 by Modified Linkage Analysis and RNAi Screens

ORAI1 was discovered in three independent genome-wide RNAi screens of drosophila S2 cells for regulators of Ca^{2+} signaling and activation of the transcription factor NFAT (Feske et al., 2006; Vig et al., 2006b; Zhang et al., 2006) and by positional cloning (Feske et al., 2006). The latter approach relied on a strategy of genome-wide linkage analysis in a pedigree with only two patients by (1) identifying potential heterozygous disease carriers through functional tests in vitro (i.e., magnitude of Ca^{2+} influx) and (2) combining two independent modes of haplotype analysis. Twelve of 21 relatives of the patients showed ~50 percent reduced Ca^{2+} influx in their T cells compared to controls, suggesting that they were heterozygous carriers of the gene defect (Fig. 20.1C). Their DNA was used for microarray-based genome-wide single-nucleotide-polymorphism (SNP) mapping followed by evaluation of SNP data in two independent linkage analyses for an autosomal recessive and dominant disease trait, respectively (Feske et al., 2006). Together, both linkage analyses yielded a combined LOD score of 5.7, defining a 6.5 Mb interval on chromosome 12q24 containing ~74 genes. The hypothetical gene locus FLJ14466 located in the candidate region was sequenced because its drosophila homolog olf-186F was among

the positive hits in a RNAi screen for NFAT activating genes (Feske et al., 2006). FLJ14466 and olf-186F were renamed ORAI1 and dOrai, respectively, after the Orai (hours) Eunomia (Harmony), Dyke (Justice), and Eirene (Peace), the keepers of heaven's gate in Greek mythology (Stewart, 2005).

Mutations in ORAI1

To date, three families with six patients have been reported to lack CRAC channel function and SOCE due to mutations in ORAI1 (Fig. 20.1A, Table 20.1). An *R91W* missense mutation in exon1 of ORAI1 was identified in two patients from the first family whose T cells lacked SOCE and I_{CRAC} (Fig. 20.1B, C). Both patients were homozygous for a C→T transition at position 271 of the ORAI1 coding sequence (NM_032790), substituting highly conserved arginine residue 91 with tryptophan at the beginning of the first transmembrane domain of ORAI1(Feske et al., 2006). Hydrophobicity of the mutant tryptophan at position 91 is essential to abolish channel function; replacement of R91 with lipophilic leucine,

Table 20.1 **PHENOTYPES OF ORAI1 AND STIM1 DEFICIENCY**

	ORAI1	STIM1
Chromosome	**12q24**	**11q15**
Gene defects (all autosomal recessive)	**R91W, A88EfsX25, A103E/L194P**	**E128RfsX9**
# of patients (families)	**6 (3)**	**3 (1)**
Clinical Symptoms		
Immunodeficiency	**Viral, bacterial, fungal infections**	**Viral, bacterial, fungal infections**
Autoimmunity	**Neutropenia (1 patient)**	**Autoimmune hemolytic anemia, thrombocytopenia, lymphoadenopathy, hepatosplenomegaly**
Congenital myopathy	**Global muscular hypotonia, atrophic type II muscle fibers (R91W), respiratory insufficiency (R91W, A103E/L194P)**	**Global muscular hypotonia**
Ectodermal dysplasia	**Enamel dentition defect: Amelogenesis imperfecta type III (R91W), anhydrosis**	**Enamel dentition defect**
Other	**Idiopathic encephalopathy Facial dysmorphy**	**Nephrotic syndrome (1 patient)**
Outcome	**Death (in first yr): 4/6 Survival after HSCT: 2/6**	**Death (1.5–9 yrs): 2/3 Survival after HSCT: 1/3**
Laboratory Findings—Immunological		
Lymphocyte counts	**Normal**	**Normal**
T, B, NK cell subsets	**Normal**	**Normal, except $CD4^+$ $CD25^+$ $Foxp3^+$ Treg ↓**
T cell activation (in vitro)	**Proliferation ↓↓ Cytokines (IL-2, IL-4, IFN-γ) ↓↓**	**Proliferation ↓-↓↓**
Immunoglobulins	**Normal—↑ Ig levels, no seroconversion**	**Normal Ig levels, no seroconversion**
Laboratory Findings—Signaling		
Protein expression	**Yes (R91W), no (A88EfsX25), no (A103E/L194P)**	**No**
SOCE/I_{CRAC}	**Absent/absent**	**Absent/not tested**

ORAI1 and STIM1 deficiency is characterized by a defect in T-cell activation resulting in immunodeficiency, congenital myopathy, and anhydrotic ectodermal dysplasia. Lack of STIM1 expression in addition is associated with hepatosplenomegaly and autoimmunity due to greatly reduced numbers of T_{reg} cells. For details see text.

HSCT, hematopoietic stem cell transplantation; I_{CRAC}, Ca^{2+} release activated Ca^{2+} (CRAC) channel current; SOCE, store-operated Ca^{2+} entry.

Data from Feske, 1996, 2000, 2006; Le Deist, 1995; McCarl, 2009; Partiseti, 1994; Picard, 2009; Schlesier, 1993.

phenylalanine, or valine, but not with charged or neutral amino acids, impaired CRAC channel function (Derler et al., 2009; McCarl et al., 2009).

A patient from a second family (Fig. 20.1A), born to consanguineous parents, lacked SOCE and CRAC channel activity (Partiseti et al., 1994) and was homozygous for insertion of a single adenine between nucleotides 258 and 259 (258insA) of the *ORAI1* coding sequence (McCarl et al., 2009). This mutation at the end of the first transmembrane domain caused a frameshift at amino acid 88 and a premature termination codon at position 112 (*ORAI1 A88EfsX25*) (Fig. 20.1A). The lack of ORAI1 mRNA and protein in this patient is consistent with nonsense-mediated mRNA decay.

Two missense mutations in *ORAI1* exon 2, A103E and L194P, were identified in an affected patient from a third family (Le Deist et al., 1995; McCarl et al., 2009) (Fig. 20.1A). Both mutations interfered with stable protein expression as no ORAI1 protein was detected in the patient's fibroblasts or in HEK293 cells ectopically expressing these ORAI1 mutants. SOCE could be reconstituted in T cells and fibroblasts from all ORAI1-deficient patients by retroviral transduction with expression vectors encoding wild-type ORAI1 (Feske et al., 2006; McCarl et al., 2009).

CLINICAL AND IMMUNOLOGICAL PHENOTYPE OF ORAI1 DEFICIENCY

The dominant clinical phenotype in all patients was immunodeficiency, with severe infections early in life, but they also had congenital myopathy and ectodermal dysplasia. Recurrent severe infections were due to viral, bacterial, mycobacterial, and fungal pathogens causing pneumonia, meningitis, enteritis, gastrointestinal candidiasis, and sepsis in the various patients (Table 20.1) (Feske et al., 1996, 2000; Le Deist et al., 1995; McCarl et al., 2009; Partiseti et al., 1994). Antibiotics and intravenous immunoglobulin (IVIg) only inefficiently controlled infections, necessitating hematopoietic stem cell transplantation (HSCT). Two of six patients were treated successfully by HSCT, but the remaining four patients died in their first year of life. ORAI1-deficient patients resembled patients with severe combined immunodeficiency (SCID), although lymphocyte counts and numbers of $CD4^+$ and $CD8^+$ T cells and of B cells were normal. T-cell activation was severely compromised, with impaired proliferation and cytokine production in response to TCR-dependent and independent stimuli in vitro and absent skin delayed-type hypersensitivity reactions in vivo (Table 20.1) (Feske et al., 1996, 2000; Le Deist et al., 1995; Partiseti et al., 1994; Schlesier et al., 1993). Despite the activation defect in vitro, increased numbers of T cells with an activated ($CD3^+$ $HLA\text{-}DR^+$) and memory ($CD4^+CD45RO^+$, $CD4^+$ $CD29^+$) phenotype were observed in the peripheral blood of all patients analyzed for these markers.

ORAI1-deficient patients also developed early global muscular hypotonia with decreased head control, delayed ambulation, and reduced muscle strength and endurance (McCarl et al., 2009). The two surviving patients after HSCT had hypotonia of respiratory muscles, chronic pulmonary disease, superinfections, and bronchiectasis by the time of adolescence. Histologically, the myopathy was characterized by a variation in muscle fiber size, with a predominance of type I fibers and atrophic type II fibers (Plate 20.1). Other structural abnormalities commonly found in congenital myopathies were not observed.

Ectodermal dysplasia with anhydrosis (EDA), impaired sweat production, and a defect in dental enamel formation occurred in both surviving ORAI1-deficient patients (Plate 20.1). Dry skin and heat intolerance led to recurrent fevers. Hypocalcified dental enamel matrix led to use-dependent loss of the soft enamel and painful exposure of underlying dentin, consistent with the diagnosis amelogenesis imperfecta type III (Plate 20.1). Scalp hair and eyebrows, often sparse or missing in other forms of EDA, were normal in ORAI1-deficient patients.

Encephalopathy observed in a patient with a ORAI1 A88EfsX25 nonsense mutation and his unaffected brother was judged unlikely to be due to ORAI1 deficiency because it occurred in only one of three families with ORAI1 defects (Partiseti et al., 1994).

STIM1 DEFICIENCY

BIOLOGY OF STIM1

The human *STIM1* gene on chromosome 11p15 consists of 12 exons. STIM1 is a single-pass transmembrane protein of 685 amino acids localized predominantly in the membrane of the ER, where it functions as a sensor of ER Ca^{2+} concentrations and activator of ORAI1/CRAC channels (Liou et al., 2005; Roos et al., 2005). STIM1 contains a pair of low-affinity EF hand calcium-binding domains, a sterile alpha motif (SAM), and two coiled-coil protein–protein interaction domains. Depletion of Ca^{2+} from the ER results in dissociation of Ca^{2+} from the N-terminal EF hand domains of STIM1, unfolding of the EF-SAM domain, and multimerization of STIM1, ultimately leading to the assembly of STIM1 in large ER membrane clusters called puncta (Liou et al., 2007; Stathopulos et al., 2008). The formation of STIM1 puncta causes aggregation of ORAI1 in the plasma membrane and localized Ca^{2+} influx (Liou et al., 2005, 2007; Luik et al., 2006, Wu et al., 2006). STIM2, a closely related paralog of STIM1, is also located in the ER, is able to heterodimerize with STIM1, and acts as a positive regulator of SOCE (Manji et al., 2000; Williams et al., 2001). STIM2 activates Ca^{2+} influx upon smaller decreases in ER Ca^{2+} concentrations than STIM1 and was shown to regulate basal cytosolic Ca^{2+} concentrations (Brandman et al., 2007).

MOLECULAR BASIS OF STIM1 DEFICIENCY

To date, three patients from one family have been reported to lack SOCE due to mutations in STIM1 (Picard et al., 2009). Patient fibroblasts showed a pronounced defect in SOCE in response to thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which induces passive

depletion of intracellular Ca^{2+} stores. Born to consanguineous parents, the proband and her younger brother were homozygous for insertion of an adenine in exon 3 of *STIM1*, resulting in a frameshift beginning at codon 128 and a premature termination codon at position 136 (E128RfsX9). Consequently, STIM1 mRNA and protein were greatly reduced or absent in the patients' cells. SOCE was restored by retroviral transduction with wild-type STIM1 while partial reconstitution was achieved by expression of STIM2, suggesting that the genes have overlapping functions. Endogenous expression levels of STIM2 were not, however, sufficient to compensate for the lack of STIM1 in the patients.

CLINICAL AND IMMUNOLOGICAL PHENOTYPE OF STIM1 DEFICIENCY

Lack of STIM1 is characterized by immunodeficiency, congenital myopathy, and ectodermal dysplasia reminiscent of ORAI1-deficient patients but in addition results in autoimmune disease (Table 20.1) (Picard et al., 2009). Patients had recurrent bacterial and viral infections such as urinary tract infections, bacterial sepsis, otitis media, and pneumonia caused by a spectrum of pathogens, including *S. pneumoniae*, *E. coli,* cytomegalovirus, and varicella zoster virus. Lymphocyte counts were slightly reduced or normal. The proband had an age-appropriate distribution of lymphocyte subpopulations and normal TCR repertoire but decreased proportions of naïve $CD4^+$ T cells and $CD4^+CD45RA^+CD31^+$ T cells (recent emigrants from the thymus). T-cell proliferation in response to stimulation with phytohemagglutinin, phorbol 12-myristate 13-acetate plus ionomycin, or anti-CD3 antibody was markedly impaired, with even less response to recall antigens.

Immunodeficiency in the STIM1-deficient patients was complicated by hepatosplenomegaly and autoimmune disease (Picard et al., 2009). Two patients had lymphadenopathy and hepatosplenomegaly, but Fas-induced T-cell apoptosis measured in one was normal. All had thrombocytopenia and two had autoimmune hemolytic anemia. A likely cause for autoimmunity in STIM1-deficient patients is their reduced number of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (T_{reg}). Mice lacking expression of STIM1 and STIM2 also show T_{reg} cell defects (Oh-Hora et al., 2008). Lack of SOCE and reduced TCR signal strength in developing T cells may cause abnormal persistence of self-reactive T cells during thymic development, leading to the autoimmunity in human patients and mice. Despite reduced numbers of T_{reg} cells, STIM1-deficient patients did not resemble patients with X-linked immune dysregulation, polyendocrinopathy, enteropathy (IPEX) syndrome (Ochs et al., 2007), presumably because of impaired antigen-specific activation of effector T cells in the absence of STIM1 and SOCE.

Like ORAI1-deficient patients, those lacking STIM1 also suffered from ectodermal dysplasia and congenital myopathy as well as partial iris hypoplasia. The myopathy is consistent with the role of STIM1 in myoblast differentiation and the defect in skeletal muscle development and function found in *Stim1*-deficient mice (Darbellay et al., 2008; Lyfenko and Dirksen, 2008; Stiber et al., 2008).

ANIMAL MODELS

Gene-targeted mice lacking *Orai1* and *Stim1* expression have been generated by homologous recombination and insertional mutagenesis. In contrast to ORAI1- and STIM1-deficient patients (Feske et al., 2006; Picard et al., 2009), mice generally die in the first days postpartum, most likely due to hypotonia. Surviving $Orai1^{-/-}$ and $Stim1^{-/-}$ mice are severely runted but may catch up with their littermates in the first weeks of life, depending on genetic background. $Stim1^{-/-}$ mice show morphological abnormalities in skeletal muscle and defects in myoblast function.

Immune function is compromised in $Orai1^{-/-}$ and $Stim1^{-/-}$ mice due to severely impaired CRAC channel function and SOCE in $CD4^+$ and $CD8^+$ T cells, B cells, mast cells, and macrophages (Baba et al., 2008; Gwack et al., 2008; Oh-Hora et al., 2008), although one study found only mild impairment of Ca^{2+} influx in *Orai1*-deficient T cells (Vig et al., 2008). As a consequence, expression of cytokines interleukin (IL)-2, interferon (IFN)-γ, IL-4, and IL-10 was substantially reduced in T cells from $Orai1^{-/-}$ and $Stim1^{-/-}$ mice (Gwack et al., 2008; Oh-Hora et al., 2008), similar to ORAI1-deficient human patients (Feske et al., 2000). By contrast, T-cell proliferation in response to TCR stimulation and T-cell-dependent antibody responses were preserved in $Orai1^{-/-}$ (Gwack et al., 2008) and $Stim1^{-/-}$ mice (Beyersdorf et al., 2009). By contrast, B cells from $Orai1^{-/-}$ mice proliferated poorly in response to BCR stimulation (Gwack et al., 2008), and mast cells from both $Orai1^{-/-}$ and $Stim1^{-/-}$ mice showed reduced cytokine secretion and degranulation in vitro and attenuated passive cutaneous anaphylaxis in vivo (Baba et al., 2008; Vig et al., 2008). Finally, macrophages lacking *Stim1* expression had severely compromised FcRγII/III-mediated Ca^{2+} influx (Braun et al., 2008) and were protected from disease in in vivo models of autoantibody-mediated thrombocytopenia and anemia. These findings suggested a role for SOCE in phagocytosis in mice (Braun et al., 2008), in contrast to autoimmune hemolytic anemia and thrombocytopenia in STIM1-deficient patients (Picard et al., 2009).

Autoimmunity, lymphadenopathy, and splenomegaly observed in STIM1-deficient patients was recapitulated in mice with conditional, T-cell-specific deletion of both *Stim1* and *Stim2* ($Stim1^{f/f}$, $Stim2^{f/f}$ *CD4-Cre*). These mice had reduced numbers and impaired function of T_{reg} cells (Oh-Hora et al., 2008). In addition, double-deficient mice showed leukocytic organ infiltration, colitis, dermatitis, and blepharitis. A potential cause for the paucity of T_{reg} cells is the failed Ca^{2+}-dependent activation of NFAT, which interacts with binding sites in the promoter and enhancer of FoxP3, the lineage-determining transcription factor of T_{reg} cells (Tone et al., 2008).

Myeloid- and lymphoid-cell development in the bone marrow and thymus was unperturbed in *Orai1-, Stim1-*, and *Stim1/Stim2*-deficient mice, consistent with the normal leukocyte numbers in ORAI1- and STIM1-deficient patients (Beyersdorf et al., 2009; Feske et al., 1996; Gwack et al., 2008; Le Deist et al., 1995; McCarl et al., 2009; Oh-Hora et al., 2008; Partiseti et al., 1994; Picard et al., 2009). Ca^{2+} signals are widely considered necessary for differentiation and

selection of T cells in the thymus, but T-cell development was normal in *Stim1*-deficient mice (*Stim1$^{f/f}$ CMV-Cre*) despite a complete lack of detectable SOCE (M. Oh-Hora, A. Rao, SF unpublished); these data suggest that STIM1 and ORAI1 may be dispensable for lymphocyte development, with the notable exception of T_{reg} cells.

PROGNOSIS AND TREATMENT

Despite normal lymphocyte development in patients lacking functional ORAI1 or STIM1, the immunodeficiency is similar in scope and severity, especially in ORAI1-deficient patients, to that of SCID patients. Four of six ORAI1-deficient patients died in their first year of life due to recurrent, severe infections, and two STIM1-deficient patients died at 1.5 and 9 years of life of encephalitis and complications of HSCT, respectively. HSCT resulted in successful immune reconstitution in two ORAI1-deficient patients and one STIM1-deficient patient. The two now-adolescent ORAI1-deficient patients, however, suffer from secondary complications of muscular hypotonia and chronic pulmonary disease. In addition, one developed a monoclonal EBV-associated polymorphic B-cell lymphoma of host origin at 8 years of age. No signs of autoimmunity were observed in the surviving STIM1-deficient patient after HSCT.

CONCLUDING REMARKS

The clinical phenotypes of ORAI1 and STIM1 deficiency largely overlap, suggesting that the developmental and functional defects are not protein-specific but rather pathway-specific—that is, that they result from the absence of SOCE and CRAC channel function. The immunodeficiency in both diseases is caused by a severe defect in T-cell activation but not T-cell development. However, STIM1, but not ORAI1, seems required for the development of $CD4^+$ $Foxp3^+$ regulatory T cells, evidenced by the reduced numbers of T_{reg} cells in the peripheral blood of one STIM1-deficient patient and autoimmune lymphoproliferative disease in all three patients (Picard et al., 2009). While numbers of T_{reg} cells could not be evaluated in ORAI1-deficient patients, symptoms of autoimmunity were apparent in only one of the patients, who had neutropenia and thrombocytopenia at 7 months of age (McCarl et al., 2009). As ORAI1-deficient patients either died in their first year of life and or were treated by HSCT, it can be speculated that a defect in T_{reg} development and subsequent autoimmunity did not have enough time to manifest in these patients. Alternatively, residual SOCE in immature T cells in ORAI1-deficient (in contrast to STIM1-deficient) patients may permit T_{reg} development. Given the expression of ORAI2 in naïve CD4+ T cells, it is conceivable that other ORAI isoforms such as ORAI2 or ORAI3 play a role in SOCE in immature T cells and that mutations in these genes may be associated with defects in T-cell development or function.

Finally, the novel strategy used to positionally clone the ORAI1 gene described in this chapter may be useful for the identification of gene defects underlying other rare autosomal recessive diseases in which traditional linkage analysis cannot yield high enough LOD scores due to the small number of affected patients. Successful application of this approach relies on (1) a reliable test to identify heterozygous carriers and (2) a large enough number of relatives who can be tested.

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21.

DEFICIENCY OF FOXN1

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FOXN1 (NUDE) DEFICIENCY

In 1996, a novel form of severe combined immunodeficiency (SCID) (MIM 601705; Pignata Gguarino syndrome) was described and proposed as the human equivalent of the well-known murine phenotype defined as Nude/SCID (Pignata et al., 1996). This syndrome represents the first example of SCID not primarily related to an abnormality of the hematopoietic cell, but rather to an intrinsic alteration of the thymic epithelial cell (reviewed in Pignata, 2002). The hallmark of the Nude/SCID mouse is congenital alopecia associated with a profound T-cell defect (Flanagan, 1966).

Human Nude/SCID was identified in two sisters who had congenital alopecia of the scalp, eyebrows, and eyelashes; nail dystrophy; and a severe T-cell immune defect, inherited as an autosomal recessive disorder (Fig. 21.1) (Pignata et al., 1996). The older sister presented at the age of 2 months with erythroderma, persistent diarrhea, failure to thrive, lymph node enlargement, and hepatosplenomegaly. Hypereosinophilia and prominent functional abnormalities of T cells but not of B cells were observed. Despite therapy the patient died of bronchopneumonia at the age of 12 months. The younger sister, evaluated at 1 month of age, showed major immunological abnormalities leading to severe respiratory infections. At 5 months, she received an allogeneic HLA-matched bone marrow transplant (BMT). Of note, 1 year following the transplant, the patient still had alopecia and nail dystrophy. Due to the similarities between the human clinical features and the mouse Nude/SCID phenotype, known to be due to a *Foxn1* defect, molecular analysis of human *FOXN1* in these girls was performed and revealed a cDNA homozygous mutation 792C>T, producing the nonsense mutation R255X in exon 5 and complete absence of a functional protein (Frank et al., 1999).

Since the first description of these Nude/SCID patients, others with a similar phenotype have been diagnosed. A Portuguese newborn with alopecia, nail dystrophy, and severe infections had the same homozygous R255X mutation as the Italian patients (M. Markert and A. E. Souza, as reported at the XIIth Meeting of the European Society for Immunodeficiencies, Budapest, 2006). Another patient born to unrelated parents of mixed French/African origin had a homozygous cDNA 987C>T (R320W) missense mutation in exon 6 of *FOXN1* (Markert et al., 2011).

CLINICAL SPECTRUM OF THE NUDE/SCID PHENOTYPE

Pathological examination of two human fetuses carrying the R255X mutation in homozygosity, identified in the geographical area where the first patients originated, revealed the lack of a thymus, confirming that Foxn1 is essential for thymic development in humans. Defects in human fetal development could explain the high rate of mortality in utero observed in kindreds of this region. Fetal skin was tighter than usual and showed basal hyperplasia and dysmaturity, suggesting impaired differentiation. (Amorosi et al., 2008). One fetus also had neural tube defects, including anencephaly and spina bifida, consistent with evidence that the mouse *Foxn1* gene is expressed in epithelial cells of the developing choroids plexus (Amorosi et al., 2008).

The T-cell defect in human Nude/SCID includes a lack of proliferative responses associated with a severe differentiation blockage of T cells (Table 21.1) (Pignata et al., 1996). A prominent decrease of $CD3^+$, $CD4^+$, $CD8^+$ cells and $CD4^+$ $CD45RA^+$ naïve lymphocytes but normal B- and NK-cell compartments are found. From immunological

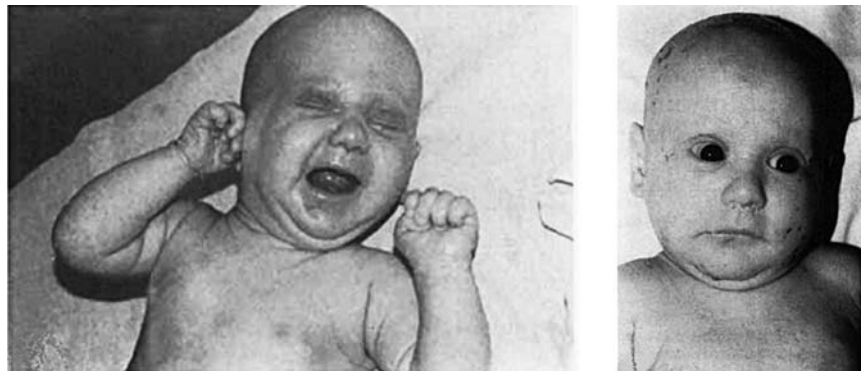

Figure 21.1 Alopecia of the scalp, eyebrows, and eyelashes, characterizing the human Nude/SCID phenotype. (From Pignata et al. *Am J Med Genet* 65:167–170, 1996, with permission.)

studies performed in a patient who underwent unmanipulated BMT, also containing mature T cells, it was documented that, despite an increase in $CD3^+$, $CD4^+$, and $CD8^+$ cells of donor origin, the $CD4^+$ $CD45RA^+$ naïve cells were not regenerated. Conversely, naïve $CD8^+$ cells increased, suggesting an extrathymic site of maturation for this subset (Pignata et al., 2001).

Heterozygous carriers of the R255X mutation frequently show congenital nail dystrophy, most specifically leukonychia, a typical arch pattern resembling a half-moon in the proximal part of the nail plate. However, the most frequent nail alteration is koilonychia ("spoon nail"), a concave surface and raised edges of the nail plate, associated with thinning of the plate itself. Canaliform dystrophy and a transverse groove of the nail plate (Beau line) may also be present (Fig. 21.2) (Auricchio et al., 2005). *FOXN1* is expressed in the nail matrix where the nail plate originates, confirming its involvement in the maturation of nails and suggesting nail dystrophy

Table 21.1 CLINICAL AND IMMUNOLOGICAL FEATURES OF HUMAN NUDE/SCID PHENOTYPE

FEATURES	NUDE/SCID
Clinical	
Athymia	Constant
Alopecia	Constant
Nail dystrophy	Constant
Failure to thrive	Frequent
Erythroderma	Frequent
Infections*	Constant
Age at presentation of infections	<6 months
Brain anomalies	Uncertain, but present in one fetus
Immunological	
Absolute lymphocyte count	Normal
$CD3^+$ cells	Markedly reduced (range: 0–25%)
$CD3^+$ $CD4^+$ cells	Absent to markedly reduced (range: 0–20%)
$CD3^+$ $CD8^+$ cells	Reduced (range: 0–11%)
$CD16^+$ $CD56^+$ cells	Normal
$CD19^+$ cells	Normal to elevated (range: 37–75%)
Naïve T lymphocytes	Extremely low (range: 0–3.1%)
Proliferative response to mitogens	Absent to markedly reduced
Specific antibody production	Absent to very low
Serum immunoglobulin levels	Normal to reduced

*Includes conjunctivitis (birth), pyogenic infections (2 mo), diarrhea (2–4 mo); BCG lymphadenitis (3 mo), *Mycobacterium bovis* infection (5 mo); severe herpesvirus 6 infection (4 mo); bronchiolitis, bronchopneumonia, interstitial pneumonitis (2–12 mo).

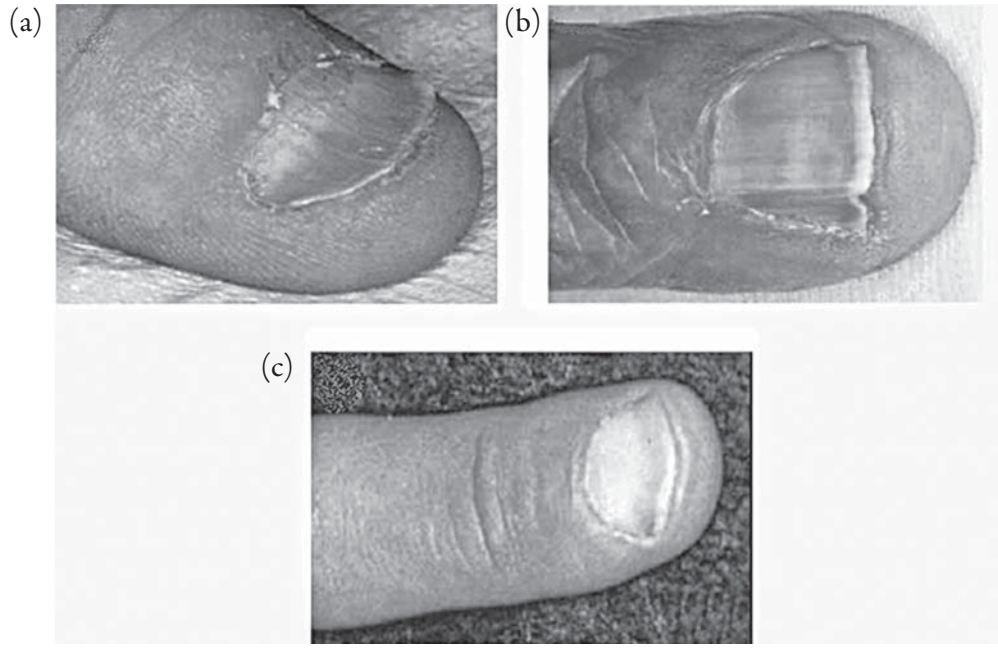

Figure 21.2 Nail dystrophy patterns associated with heterozygous *FOXN1* mutation: (A) koilonychia, (B) canaliform dystrophy, (C) leukonychia. (From Auricchio et al. *Arch Dermatol* 141:647–648, 2005, with permission.)

as a sign of heterozygosity for *FOXN1* mutation (Auricchio et al., 2005).

THE MURINE NUDE PHENOTYPE

In 1966, S. P. Flanagan identified a mouse phenotype that spontaneously appeared in the Virus Laboratory of Ruchill Hospital of Glasgow. The mouse had hair fibers with abnormal keratinization and a follicular infundibulum unable to enter the epidermis (Flanagan, 1966). Affected mice also showed dysgenesis of the thymus (Pantelouris et al., 1970; Takahashi et al., 1992), resulting in a compromised immune system lacking T cells. The analysis of serial histological sections of the area where a normal thymus would be found revealed absence of thymic epithelial cells, Hassall's corpuscles, or thymocytes. However, occasionally in the anterior mediastinum an irregularly shaped, minute mass containing normal and brown fat was present. Lymph nodes and Peyer's patches revealed a dramatic lymphoid depletion of the paracortical areas. A marked depletion of cells was also found in the periarteriolar region (the presumed thymic-dependent area) of the spleen.

Nude mice have a severe T-cell deficiency and an overall severely impaired immune response. Lymphocytes are unable to respond to T-cell mitogens or antigens. IgM levels are elevated, IgG1 and IgG2 mildly depressed, and IgA are undetectable (Amman et al., 1971; Bankhurst et al., 1975). Nude mice possess T-cell precursors, and cell-mediated immunity can be partially restored by either thymic extracts or thymus transplantation (Gershwin et al., 1975; Loor et al., 1973). It should be mentioned that studies performed in Nude/SCID mice gave a great contribution to the knowledge of cell-mediated immunity. For a long time, the DiGeorge syndrome (DGS) was erroneously considered the human counterpart of the murine Nude/SCID phenotype, although the immunological impairment profoundly differs between DGS and mouse Nude/SCID.

The molecular nature of the nude defect was characterized and attributed to deficiency of the transcription factor Foxn1, also called Whn or Hfh11 (Byrd, 1993; Nehls et al., 1994; Takahashi et al., 1992). Spontaneous mutations in Foxn1 also occur in rats (Nehls et al., 1994) and result in a comparable phenotype (Cash et al., 1993).

The Nude mouse epidermis shows failure in differentiation and reduced number of tonofilaments in spinous, granular, and basal layers (Köpf-Maier et al., 1990). The nude *Foxn1* gene doesn't affect the growth of hair follicles, but rather the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle (Brissette et al., 1996; Lee et al., 1999). Alterations of digits and nails were also reported in a few strains of Nude mice, probably due to an abnormal production of filaggrin protein in the nail matrix and nail plate, subsequent to a loss of keratin 1 protein. Increased fetal loss may be due to a change in the hormonal status, as demonstrated by altered serum levels of estradiol, progesterone, and thyroxine (Köpf-Maier et al., 1990).

FOXN1 AND ITS ROLE IN THYMUS AND SKIN

Foxn1 is a highly conserved transcription factor. It belongs to the forkhead gene family that comprises a diverse group of "winged-helix" transcription factors that are involved in

development, metabolism, cancer, and aging. These factors are developmentally regulated and direct tissue-specific transcription and cell-fate decisions (Lai et al., 1993). Foxn1 expression is regulated by wingless (Wnt) proteins (Balciunaite et al., 2002) and bone morphogenetic proteins (BMPs) (Tsai et al., 2003) in both autocrine and paracrine fashions (reviewed in Jonsson et al., 2005), and its expression is restricted to epithelial cells in the skin and thymus (Brissette et al., 1996). During embryogenesis in mice Foxn1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract as well as skin and thymus. In adult life its expression is limited to epithelial cells of the intestine, spermatocytes, and thymus (Ye et al., 1997). In particular, on the basis of the observation that Nude mouse keratinocytes do not differentiate in a normal fashion, Foxn1 could be considered as a key regulator of the balance between keratinocyte growth and differentiation, through its ability to suppress involucrin and locrin, both components of the cornified envelope, and profilaggrin, involved in the aggregation of the intermediate filaments.

The expression of Foxn1 begins in the mouse thymus anlage at about E11.5. Within a normal thymus, mature T lymphocytes derive from the interaction between the thymic epithelial cell, representing the main component of the stroma, and the T-cell precursors (thymocytes) (reviewed in Blackburn et al., 2002, and Manley, 2003). Thymic epithelial cells are involved in both thymus organogenesis and most of the maturation stages of thymocyte development.

Of note, there are remarkable similarities between thymic epithelial cells, keratinocytes, and skin fibroblasts that led to consideration of the skin as a nonhematopoietic tissue able to support the development of functional human T cells. Foxn1 is expressed in human skin cell/hematopoietic progenitor cell (HPC) cultures, where the full process of human T-cell development can be modeled (Clark et al., 2005). Although thymus and skin are different in three-dimensional structure, experiments performed with keratinocytes and fibroblasts of the skin and HPCs obtained from bone marrow, reconfigured in a different three-dimensional arrangement, demonstrate the capacity to generate T cells with the hallmarks of recent thymic emigrants, such as the presence of T-cell surface markers including the CD3/T-cell receptor (TCR) complex (Douek et al., 1998) and TCR excision circles, which derive from the recombination of TCR genes. These cells also possess a diverse TCR repertoire and can be considered mature and functional because they have full capacity to proliferate, express the activation antigen CD69, and produce cytokines in response to TCR/CD3 stimulation. Therefore, skin and bone marrow elements might be used to generate de novo functional and diverse T-cell populations, thus opening new windows for innovative therapeutic approaches to immunological disease (Clark et al., 2005).

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22.

CHRONIC MUCOCUTANEOUS CANDIDIASIS AND SUSCEPTIBILITY TO FUNGAL INFECTIONS DUE TO DEFECTS IN CARD9 AND DECTIN-1

Bodo Grimbacher

Chronic mucocutaneous candidiasis (CMC) comprises a wide range of disorders characterized by persistent and recurrent infections of the skin, nails, and mucosa with predominantly *Candida albicans*. CMC is a heterogeneous disease and may be complicated by endocrinopathies such as hypoparathyroidism, hypoadrenalism, and ovarian failure as well as autoinflammatory disorders including autoimmune hepatitis, vitiligo, and alopecia (Kirkpatrick, 2001; Lilic, 2002). Additionally, CMC patients may be susceptible to infections with pathogens other than *C. albicans*. Bacterial infections of the respiratory tract, urogenital tract, and skin have also been described (Kirkpatrick, 2001) as well as invasive fungal infections with *Histoplasma capsulatum* or *Cryptococcus neoformans*; CNS infections due to *Candida* or *Cryptococcus* are rare but have been reported (Kauffman and Shead, 1981; Kirkpatrick, 2001; van 't Wout et al., 1988). CMC patients may also present with dermatophyte infections of the skin and nails, often resulting in disfigurements (Kirkpatrick, 2001; Shama and Kirkpatrick, 1980). The majority of CMC patients present in childhood with stubborn and persistent *Candida* infection of the mouth, known as thrush, and the diaper area. The infection is usually localized but may spread and affect nails and other skin areas. In adults with late onset, a thymoma with or without thymoma-associated diseases such as myasthenia gravis may be present (Kirkpatrick, 2001; Kirkpatrick and Windhorst, 1979; Lilic, 2002).

CMC is a primary immunodeficiency that must be distinguished from APECED (autoimmune polyendocrinopathy candidiasis ectodermal dysplasia), also known as APS1 (autoimmune polyglandular syndrome type 1), caused by mutations in the *AIRE* (autoimmune regulator) gene and in which candidiasis is a clinical hallmark (see Chapter 31) (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997). Other primary immunodeficiencies such as severe combined immunodeficiency (SCID) (Chapter 9), DiGeorge syndrome (Chapter 45), or hyper-IgE syndrome (Chapter 38) must be considered, as well as secondary immunodeficiency due to underlying diseases including iron deficiency, HIV infection, diabetes mellitus, or denture stomatitis (Kirkpatrick, 2001). The long-term use of immunosuppressive drugs, in particular inhaled steroids, is also associated with oral candidiasis.

Increased susceptibility to mucosal and cutaneous *Candida* and other fungi is often attributed to an impaired cellular immune response and may be observed in patients with congenital or acquired T-cell defects. A wide range of different immunological abnormalities in T cells of CMC patients has been described (de Moraes-Vasconcelos et al, 2001; Eyerich et al., 2007, 2008; Kirkpatrick, 2001; Lilic et al., 2003). A critical factor for the pathogenesis may be the impaired ability of T cells to release certain cytokines, with a shift from typical Th1 cytokines such as interleukin (IL)-12 to IL-10, but impaired maturation of dendritic cells may also contribute to the disease in CMC patients with or without APECED/APS1 (Ryan et al., 2008).

A subset of T cells, IL-17 and IL-22 producing helper T cells, is required for adaptive antifungal immunity. These Th17 cells play a crucial role in the host defense against *Candida* in mice, and these cells are reduced in number in patients with CMC (Conti et al., 2009; Eyerich et al., 2008; Glocker et al., 2009; LeibundGut-Landmann et al., 2007). The majority of CMC patients have normal serum immunoglobulin levels and high titers of anti-*Candida* antibodies. In patients with CMC and additional severe bacterial infections such as pneumonia, a careful examination of their immune status should be carried out to clarify the extent of immunodeficiency. Most cases of CMC are sporadic, but families with both autosomal dominant and recessive inheritance have been reported (Atkinson et al., 2001; Loeys et al., 1999; Sams et al., 1979; Wells et al.,

19972). Genetic studies found linkage of a dominant candidiasis/thyroiditis syndrome to chromosome 2 and candidiasis associated with low ICAM-1 expression to chromosome 11, but no causative genes have been identified (Atkinson et al., 2001; Mangino et al., 2003).

Genetic and functional studies in a large consanguineous five-generation family identified four CMC patients carrying a homozygous loss-of-function mutation in the gene *CARD9* encoding the caspase recruitment domain-containing protein 9 (Glocker et al., 2009). The gene, located on chromosome 9, encodes a protein of 536 amino acids. Like other CARD proteins, CARD9 has an N-terminal CARD domain that mediates binding to other CARD-domain containing molecules, and a C-terminal coiled-coil domain enabling protein oligomerization. The null mutation detected in the patients results in a premature termination codon at amino acid 295 (Q295X) (Glocker et al., 2009).

CARD9 plays a central role in human antifungal defense by triggering proinflammatory responses (Fig. 22.1). The transmembrane pattern recognition receptor Dectin-1 senses 1–3 and 1–6 linked β-glucans, fungal pathogen associated molecular patterns, and signals via an immunoreceptor tyrosine-based activation (ITAM) motif that becomes phosphorylated by sarcoma (Src) kinases (Gross et al., 2006; Ruland, 2008; Taylor et al., 2007). The phosphorylated ITAM recruits and activates the SH2 domain containing spleen tyrosine kinase (SYK). The Dectin-1/SYK complex can then engage CARD9, which associates with B-cell leukemia/lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue 1 (MALT1) to form an intracellular signaling structure that relays the fungal recognition signal. The downstream elements in this pathway include the transcription factor nuclear factor kappa B (NF-κB), as well as p38 mitogen activated protein kinase (MAPK) and JNK kinase (Fig. 22.1). This signaling pathway is operative in myeloid cells and triggers the production of key cytokines such as TNF-α, IL-1β, IL-6, and IL-23 (LeibundGut-Landmann et al., 2007; Ruland, 2008), which are required for innate antifungal immunity and the generation of Th17 cells (Fig. 22.2). In addition to Dectin-1, Dectin-2 and Mincle may also recognize fungi, engage the ITAM adaptor FcRγ for SYK activation, and signal via the CARD9 pathway (Sato et al., 2006; Yamasaki et al., 2008).

By using an in vivo model based on cells from Card9$^{-/-}$ mice, it was demonstrated that the human wild-type CARD9 protein, but not the truncated CARD9 protein of patients carrying the Q295X mutation, reconstituted Dectin-1 mediated signaling in murine Card9$^{-/-}$ macrophages (Glocker et al., 2009). In parallel to *Card9*$^{-/-}$ mice, all patients with mutated CARD9 had reduced numbers of Th17 cells, compounding their increased susceptibility to fungal infections. This supports the observations that (1) CARD9-mediated signaling contributes to Th17 cell differentiation and (2) Th17 cells play a critical role in the defense against *Candida* in CMC patients (Conti et al., 2009; Eyerich et al., 2008).

The importance of Dectin-1 as pathogen recognition receptor (PRR) has been demonstrated not only in dectin-1–deficient mice, which are susceptible to infections with *C. albicans*, but also in human patients with a single point mutation in Dectin-1 leading to a premature stop codon, defective surface expression, and a reduced production of TNF, IL-6, and IL-17 (Ferwerda et al., 2009; Taylor et al., 2007). These patients suffered from recurrent vulvovaginal candidiasis and onychomycosis. Remarkably, there was no impact on fungal phagocytosis and intracellular killing (Ferwerda et al., 2009).

Thus, these rare human genetic deficiencies of Dectin-1- and CARD9 with susceptibility to fungal infections emphasize the need for intact Dectin-1/CARD9 signaling to maintain adequate antifungal defenses. Since candidiasis is relatively common, genetic defects impairing the Dectin-1/CARD9 signaling pathway may underlie susceptibility to fungal infection more frequently than previously appreciated.

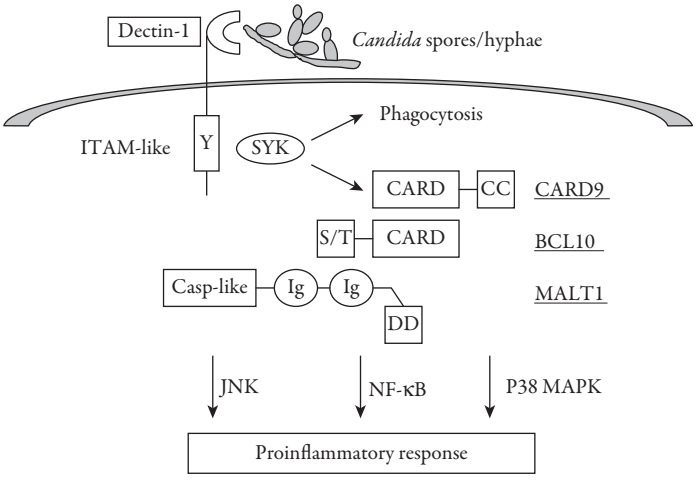

Figure 22.1 Summary of Dectin-1/CARD9 signaling pathway (modified after J. Ruland). CC, coiled-coil domain; S/T, serine/threonine-rich region; Ig, immunoglobulin repeat; DD, death domain.

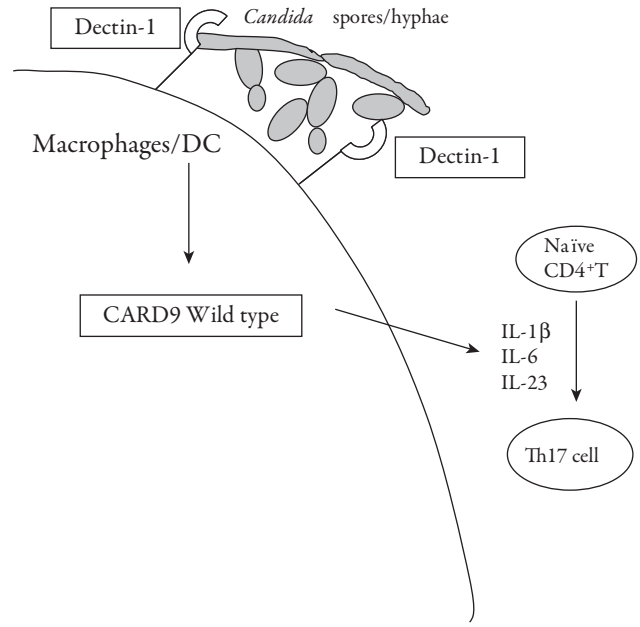

Figure 22.2 Generation of cytokines required for differentiation of naïve T cells toward Th17 cells. DC, dendritic cells.

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23.

SEVERE COMBINED IMMUNODEFICIENCY DUE TO ABSENT CORONIN-1A

Lawrence R. Shiow, Kenneth Paris, and Jennifer M. Puck

INTRODUCTION

The actin regulator Coronin-1 (also known as tryptophan-aspartate containing coat protein or TACO, ClipinA, or p57) is a cytoplasmic actin-binding protein predominantly expressed in hematopoietic cells (Su et al., 2004). Coronin-1A also binds to and inhibits the actin nucleating complex Arp2/3, which is critical for actin cytoskeleton branching. Normal T lineage cells express Coronin-1A, but lack other coronin family proteins with redundant function. Thus Coronin-1A deficient T-cells in both human and mouse are unable to remodel their actin cytoskeleton and consequently have impaired survival, signaling, and migration. B and natural killer (NK) cells are largely spared. Coronin-1A deficiency in humans (MIM #6050000) causes T lymphocyte impairment that may result in moderate combined immunodeficiency (CID) to severe combined immunodeficiency (SCID) similar to the murine ortholog (Shiow et al., 2008, 2009; Moshous et al., 2013).

CORONIN-1A STRUCTURE AND MOLECULAR FUNCTION

The *CORO1A* gene contains 11 exons and is located on human chromosome 16p11.2, a locus enriched with segmental duplications and prone to copy number variation (Martin et al., 2004; Stankiewicz et al., 2002). Coronin-1A is a 57 kDa protein with WD repeat domains near the N-terminus and a C-terminal coiled-coil domain. There is 95 percent amino acid sequence homology between human and mouse (Altschul et al., 1990). Coronin-1A WD repeat domains form a seven-bladed beta-propeller (Appleton et al., 2006). A conserved arginine residue near the N-terminus, displayed on the surface of the propeller, is required for binding to filamentous (F)-actin (Cai et al., 2007). The coiled-coil domain is thought to mediate homo-oligomerization of Coronin-1A (Oku et al., 2005). The molecular role of Coronin-1A in actin remodeling is similar to that of closely related and more ubiquitously expressed Coronin-1B. Both Coronin-1A and Coronin-1B share a conserved N-terminal phosphorylation site that regulates Arp2/3 binding (Cai et al., 2005, 2008; Foger et al., 2006).

Coronin-1A was co-purified with *phox* proteins responsible for generating reactive superoxides for phagosomes (Grogan et al., 1997). In *Mycobacterium bovis*-infected human macrophages, Coronin-1A was associated with F-actin at early phagosomes (Schuller et al., 2001). In a human leukemic T cells and mouse lymphocytes and macrophages, it co-localized with F-actin near the leading edge during cell migration and clustered with cross-linked T-cell receptor complexes (Combaluzier et al., 2009; Ferrari et al., 1999; Foger et al., 2006; Haraldsson et al., 2008; Jayachandran et al., 2007; Mueller et al., 2008; Mugnier et al., 2008; Nal et al., 2004; Shiow et al., 2008).

CLINICAL, IMMUNOLOGICAL, AND MOLECULAR FINDINGS

The first human with Coronin-1A deficiency was a girl who suffered from recurrent infections in her first year of life and had severe varicella following immunization with live attenuated varicella vaccine at age 13 months (Table 23.1) (Shiow et al., 2008, 2009). A chest CT scan showed a normal-sized thymus, unusual for SCID and similar to patients with interleukin-2 receptor alpha deficiency (Roifman, 2000), suggesting that the defect primarily affects thymocytes after the double-positive developmental stage. Indeed, this feature was also consistent with mouse models of Coronin-1A deficiency. The patient had reduced T-cell but normal B- and

Table 23.1 CLINICAL AND IMMUNOLOGICAL FEATURES OF CORONIN-1A DEFICIENCY

Growth and Development	Failure to thrive (Speech and motor delay, behavioral difficulties in one patient with concomitant interstitial deletion of chromosome 16p.11.2.)
Infections	Severe post-vaccination varicella Severe rotaviral diarrhea Recurrent otitis media Chronic pansinusitis Chronic pneumonia Oral thrush
Thymus	Present, normal in size
Lymphocyte Phenotype	Reduced CD3 T-cell number, B and NK cells present
Mitogenic Challenge	Reduced responses
Immunoglobulins	Normal total IgM and IgG Poor antibody responses to immunizations Poor primary and secondary responses to ΦX-174

NK-cell numbers. Proliferative responses to mitogens were low. While total serum immunoglobulin levels were normal, specific antibody responses to tetanus and pneumococcal antigens were poor. The patient also had a poor primary response and almost no secondary response when challenged with bacteriophage ΦX-174. Later in childhood she also had low-average to average cognitive functioning and attention-deficit/hyperactivity disorder (ADHD) that responded to stimulant medication.

Sequencing of genomic DNA revealed the patient had inherited from her father a 2 bp deletion in *CORO1A* at cDNA 248–249 (Shiow et al., 2008). This frameshift mutation resulted in a premature stop codon within the beta-propeller, predicted either to produce unstable mRNA or to destabilize protein folding. Coronin-1A protein was absent when assayed by Western blot and flow cytometric analysis. Maternal DNA showed a normal *CORO1A* sequence, but a genomic copy number microarray analysis (see Chapter 57; Fig. 57.2) determined that the patient had a de novo 600 kb deletion of her maternal chromosome 16p11.2, making her hemizygous for *CORO1A* (Shiow et al., 2009). The deleted interval, flanked by nearly identical segmental duplications, is known to be susceptible to copy number variation, with deletions and duplications associated with autism spectrum disorder and neurodevelopmental conditions, including ADHD (Kumar et al., 2008; Weiss et al., 2008).

Coronin-1A deficiency was also found by whole-exome sequencing in 3 siblings with intragenic mutations who exhibited similar immunodeficiency to that of the index case (Moshous et al., 2013).

TREATMENT

Despite medical management with antibiotics and intravenous immunoglobulin ,the original patient had failure to thrive with recurrent and progressive infections, particularly of the lungs. At 4 years of age she received a matched unrelated cord blood transplant resulting in full myeloid and lymphoid donor chimerism and complete immune reconstitution. By 6.5 years of age, she exhibited accelerated growth and resolution of pulmonary infiltrates. The three consanguimrous siblings all had T cell lymphopenia. While the oldest survived after treatment at 1 year of age for acular EBV-related orbital lymphoma, the other siblings, who developed B cell lympho Proliferation at 7.5 and 14 months, died from complications of chemotheraphy and hematopoietic cell transplantation, respectively. These individuals had a hemozygous V134M missense mutation that deerwassed, but did not abregate Coronin-1A activity.

MOUSE MODELS

Targeted disruption or hypomorphic mutations of Coronin-1A in mice result in T lymphocytopenia with sparing of B and NK cells (Foger et al., 2006; Haraldsson et al., 2008; Mueller et al., 2008; Mugnier et al., 2008; Shiow et al., 2008). In agreement with the thymus observed in the human patient, these mice have normal thymocyte numbers. Murine Coronin-1A deficiency results in late thymocyte developmental arrest at the mature single-positive stage (Foger et al., 2006) or impaired TCR signaling (Mueller et al., 2008; Mugnier et al., 2008). Thymocytes and T cells from Coronin-1A–defective mice also display impaired cellular migration to chemotactic cues (Foger et al., 2006; Shiow et al., 2008). The T cells display abnormal cellular protrusions when migrating in vitro and have impaired migration when tracked by two-photon microscopy of explanted lymph nodes (Shiow et al., 2008).

While Coronin-1A deficiency does reduce B-cell numbers, T-dependent antibody responses and germinal center formation are impaired (Combaluzier et al., 2009; Haraldsson et al., 2008; L. Shiow, unpublished observations). However, T-independent antibody production is not affected, suggesting that Coronin-1A is not critical for B-cell signaling. Redundancy with other coronin family members may explain why B, NK, and other leukocyte subsets do not require Coronin-1A for survival, signaling, and migration.

CONCLUSIONS AND FUTURE DIRECTION

Coronin-1A deficiency is the first SCID disease to be associated with actin cytoskeleton regulation. With its nonredundant and essential role in T-cell homeostasis and the *CORO1A* gene localization within a copy number variant locus, future cases of Coronin-1A deficient T^- B^+ NK^+ SCID are expected. Future studies of human T cells lacking Coronin-1A may shed light on how this molecule promotes cell survival. Further biochemical work on related coronins and studies in mouse models may further clarify mechanisms of actin cytoskeleton regulation. For example, Coronin-1A–deficient T cells

display varying degrees of TCR signaling abnormalities, failing to maximally flux calcium after exposure to anti-CD3 (Haraldsson et al., 2008; Mueller et al., 2008; Mugnier et al., 2008; Shiow et al., 2008). Analogously, in vitro studies with Coronin-1A–deficient B cells have demonstrated an antigen receptor-signaling defect that resolved with stronger co-stimulation (Combaluzier et al., 2009). Future work is needed to determine whether antibody defects reflect a primary role for Coronin-1A or are secondary to impaired T-cell help.

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24.

BRIEF INTRODUCTION TO B-LYMPHOCYTE DEFECTS

C. I. Edvard Smith

The B-cell lineage is affected in many forms of primary immunodeficiencies. Often this is due to a defect inherent to the B cell itself, but in an even greater number of disorders it is secondary to abnormal T lymphocytes, which cannot provide adequate signals for B lymphocytes to thrive. Thus, as a rule, altered B-cell populations accompany T-lymphocyte abnormalities.

The most classical B-lineage defect is X-linked agammaglobulinemia (XLA), which was first reported by Ogden C. Bruton in 1952 and was later shown to be caused by a differentiation defect, where patients essentially lack B lymphocytes and plasma cells (Chapter 25). In XLA the defect is inherent to the B-cell lineage. Because the disease gene is located on the X chromosome, XLA accounts for the majority of defects affecting pre-B-cell receptor (pre-BCR)/BCR signaling. These patients are highly susceptible to infections with pyogenic bacteria but also to enteroviruses, which in the healthy individual may be removed by circulating antibodies, but also following binding to immunoglobulins on the surface of B lymphocytes.

Over the past years, an increasing number of rare, autosomal recessive (AR) forms, currently affecting five different components of the pre-BCR/BCR, have been identified. These defects are described in Chapter 25 and are listed in Table 1.1 ("B. Deficiencies Predominantly Affecting Antibody Production"). Phenotypically they are often indistinguishable from the X-linked form but can be revealed through their different inheritance. A recently identified AR form of agammaglobulinemia is deficiency of the p85α subunit of PI3-kinase (Conley et al., 2012).

Mutations in the genes encoding the constant region of the heavy and kappa light chains, described in the 1980s, caused the first known immunodeficiencies specifically affecting the B-cell lineage. They are listed in sections B.2 and B.3, respectively, in Table 1.1. In contrast to patients lacking B lymphocytes, many individuals with heavy chain defects are healthy. The genetic abnormality is most often caused by gene deletions, and the absence of one gene product frequently seems to result in increased expression of other isotypes, likely having a compensatory effect. Because these defects only occasionally cause clinically manifest disease, they are not further described in any of the chapters.

The most common B-lineage defects are IgA deficiency and common variable immunodeficiency (CVID), described in Chapter 28. The underlying mechanisms remain elusive, although certain predisposing genes have been described. In particular, the *TACI* gene has been extensively studied in very large patient materials. Over the past few years, CVID-related diseases affecting genes encoding a B-cell–expressed CD (cluster of differentiation) marker have been described, namely those involving CD19, CD20, and CD81 (Chapter 28; see also Table 1.1, section B). A syndrome characterized by cold urticaria, and where 11 percent of patients also are diagnosed as having CVID, was recently reported (Ombrello et al., 2012) to originate from internal, in-frame deletions, resulting in a truncated, constitutively active form of phospholipase Cγ2 (Table 1.1; see the CVID section B.4). While it is likely that such mutations are rare, this points to the fact that highly unusual phenotypes may result from internal deletions also in other genes, considerably increasing the total number of immunodeficiencies. Complete inactivation of the *PLCG2* gene has not been reported to date, but the outcome, based on mouse studies, suggests that it may result in a phenotype severely affecting B-lymphocyte development. Similar to the effect of the internal deletion, it is likely that the phenotype is not restricted to the B-cell lineage (Faccio and Cremasco, 2010).

A reduced number of B cells expressing CD27, a "memory cell" marker, is a hallmark of patients with CVID, but the

underlying mechanism is not related to abnormalities in the *CD27* gene itself. Recently, however, patients with mutations in the gene for CD27 were found to suffer from severe Epstein-Barr virus (EBV) infections (van Montfrans et al., 2012). The defect does not seem to primarily impair B-lymphocyte function, but rather to affect the cellular immunity. For this reason CD27 deficiency is listed in Table 1.1 under the section "E.8 Familial hemophagocytic lymphohistiocytosis (FHL) and lymphoproliferative syndromes." These syndromes are also mentioned in Chapter 1, since they demonstrate that human beings are equipped with several genes to provide protection against otherwise lethal EBV infections.

Of great interest for B-cell biology are the defects of class-switch recombination and somatic hypermutation (Chapters 26 and 27). These disorders were initially named "dysgammaglobulinemia," with deficiency of 7S and elevated 19S gammaglobulins, and subsequently "hyper-IgM syndromes," because when first described in the early 1960s, the characteristic phenotype was one of increased serum IgM. However, while inefficient class-switching may cause this laboratory abnormality, the symptoms are secondary to the lack of IgA, IgG, and IgE, which cannot be expressed owing to the primary defect. Importantly, once the corresponding disease genes were cloned, it became immediately apparent that many affected patients present with hypogammaglobulinemia and normal or low serum IgM. So, even if the original name is still frequently being used, it should be replaced by the gene defect. Moreover, while all class-switch recombination abnormalities by definition affect B lymphocytes, the "classical" class-switch immunodeficiencies caused by mutated genes for the CD40 Ligand (CD40L) or CD40 primarily impair T-lymphocyte function. The affected patients are prone to develop opportunistic infections and malignancies, resulting in a more severe phenotype; they are listed in Table 1.1, section A.8. On the other hand, class-switch recombination syndromes affecting only B cells, exemplified by mutations in the AID and UNG genes, are generally milder and discussed in Chapter 27 (see section B.6 of Table 1.1).

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25.

X-LINKED AGAMMAGLOBULINEMIA AND AUTOSOMAL RECESSIVE AGAMMAGLOBULINEMIA

C. I. Edvard Smith and Mary Ellen Conley

X-linked agammaglobulinemiac (XLA) and the known forms of autosomal recessive agammaglobulinemia (ARA) are caused by a B-lymphocyte differentiation arrest. XLA was originally described in 1952 by the American physician Dr. (Colonel) Ogden C. Bruton (Bruton 1952). It is frequently recognized as the prototype primary immunodeficiency (Rosen et al., 1984); it was one of the first immunodeficiencies described and it was clearly the first for which a laboratory finding (absence of the gammaglobulin fraction on protein electrophoresis) dictated effective therapy (gammaglobulin). Both XLA and ARA are characterized by an increased susceptibility to extracellular bacterial infections (Conley et al., 2009; Lederman and Winkelstein, 1985; Lopez-Granados et al., 2002; Ochs and Smith, 1996; Sideras and Smith, 1995). Enteroviral infections also frequently run a severe course, often resist therapy, and may result in significant morbidity or death (Lederman and Winkelstein, 1985; Lopez-Granados et al., 2002; McKinney et al., 1987; Misbah et al., 1992; Ochs and Smith, 1996; Plebani et al., 2002, Winkelstein et al., 2006). Infections with mycoplasmas and giardia also occur more frequently (Franz et al., 1997; LoGalbo et al., 1982; Roifman et al., 1986).

Through different approaches, the gene affected in XLA, *BTK* (MIM 300300), was isolated simultaneously by two groups and found to encode a novel cytoplasmic tyrosine kinase designated *Bruton's agammaglobulinemia tyrosine kinase*, or BTK (Tsukada et al., 1993; Vetrie et al., 1993).

Mutations in the μ heavy chain gene (*IGHM*, MIM 147020), the first gene to be associated with ARA, were reported 3 years later (Yel et al., 1996) in seven patients who had a disorder very similar to XLA. Based on the hypothesis that mutations in other genes encoding components of the B-cell receptor (BCR) might cause agammaglobulinemia, mutations in the genes for λ5 (*IGLL1* MIM 146770), Igα (*CD79A* MIM 112205), and Igβ (*CD79B* MIM 147245) were sought and identified (Dobbs et al., 2007; Ferrari et al., 2007; Minegishi et al., 1998, 1999a). Mutations in the gene for the scaffold protein BLNK, which assembles signaling molecules downstream of the BCR, have also been associated with agammaglobulinemia (*BLNK* MIM 604515) (Minegishi et al., 1999c). On the whole, the different forms of ARA are phenocopies of XLA, although the patients with ARA tend to come to medical attention earlier and are more likely to have severe complications of agammaglobulinemia (Conley et al., 2005a; Lopez-Granados et al., 2002).

Bruton's initial report described an 8-year-old boy highly susceptible to bacterial infections, in particular *Streptococcus pneumoniae*, from the age of 4.5 years (Bruton, 1952a). As reviewed in greater detail (Sideras and Smith, 1995; Smith and Notarangelo, 1997), the electrophoretic analysis of serum, which had only recently been applied in a clinical setting, revealed the absence of detectable immunoglobulins (Ig). This finding prompted Bruton to coin the term *agammaglobulinemia*, a name that has been kept in spite of the observation that minute and sometimes even considerable amounts of Ig are detectable in some XLA patients. Bruton not only realized that there was a connection between the absence of Ig and the susceptibility to infections, he also initiated substitution treatment with subcutaneous γ-globulin and demonstrated its efficacy.

It has been noticed that the patient initially described by Bruton had some characteristics that are not typical for classical XLA (reviewed in Sideras and Smith, 1995). However, certain patients with XLA, as confirmed by mutation analysis, have a mild disease phenotype similar to, or even milder than, that of Bruton's case (Broides et al., 2006; Bykowsky et al., 1996; Jones et al., 1996; Hashimoto et al., 1999; Plebani et al., 2002; Saffran et al., 1994; Stewart et al., 2001; Vihinen et al., 1995a, 1996b, 2001; Wood et al., 2001). In a collaborative study, Bruton et al. reported on additional patients in whom the diagnosis of XLA seems indisputable (Bruton et al., 1952); more importantly,

the original report inspired a great number of investigators to look for patients with antibody deficiency. As a result, within a few years, many patients were identified worldwide not only with XLA but also with many other forms of Ig abnormality (reviewed in Good et al., 1962; Sideras and Smith, 1995). The recognition of different forms of antibody deficiency disease was a gradual process, and it was not until the late 1960s and early 1970s, when B cells were first recognized to express cell-surface Ig, that a classification as we know it today was generally accepted (Cooper et al., 1973; Naor et al., 1969).

Both XLA and ARA are characterized by an increased susceptibility to infections, with onset most often during the first year of life, when transferred maternal Ig has been catabolized. Analysis of serum demonstrates a pronounced decrease in Ig levels of all isotypes; IgA is usually undetectable. There is a virtual absence of humoral response to recall antigens. B-lymphocyte and plasma-cell numbers are markedly decreased, whereas other cell lineages are usually normal in number and phenotype. The defects in XLA and ARA cause a differentiation arrest confined to the B-cell lineage (Fig. 25.1), distinguishing XLA and ARA from several other Ig deficiencies. Collectively these mutations dampen or extinguish signaling from the pre-BCR/BCR resulting in similar phenotypes. B-lineage cells in all organs are affected, resulting in a reduced size of lymph nodes and tonsils. Approximately 85 percent of patients with lack of B lymphocytes and low, or absent, levels of Ig have mutations in the *BTK* gene (Conley et al., 1998). Another 5 percent have mutations in μ heavy chain, 4 to 5 percent have defects in λ5, Igα, Igβ, or BLNK, and the nature of the gene defect remains unknown in about 5 percent of patients (Conley et al., 2005b).

The defective gene in XLA encodes a cytoplasmic tyrosine kinase designated BTK in humans (and Btk in mice). The genes causing ARA, when mutated, are presented in Chapter 1, Table 1.1, Section B: Deficiencies Predominantly Affecting Antibody Production. Mutation analysis will confirm the diagnosis and may be used to identify healthy carriers. Treatment consists of antibiotics to combat ongoing infections and prevent infections and γ-globulin substitution as prophylaxis.

CLINICAL MANIFESTATIONS

INCIDENCE

The incidence of XLA has been investigated in several ethnic populations (Sideras and Smith, 1995; Toth et al., 2009; Winkelstein et al., 2006). In the United States the calculated frequency is at least 1/379,000 (Winkelstein et al., 2006). The possibility that there are ethnic differences in the incidence of XLA has been considered. In a Japanese survey by Hayakawa et al. (1981) only 56 XLA cases were reported. Low numbers were also found in Malaysia (Noh et al., 1995), in black populations in the United States and South Africa, as well as in an Eastern and Central European population (Toth et al., 2009). However, lethal X-linked traits are believed to occur by de novo mutations in approximately one third of the cases (Chase and Murphy, 1973; Haldane, 1935), a finding arguing against variations in mutation frequency among different ethnic populations. Rather, underreporting is more likely. In support of this notion, the number of Japanese patients with verified mutations in the *BTK* gene has increased considerably (Kanegane et al., 2001). However, delayed diagnosis in non-Caucasians has been reported (Conley and Howard, 2002) and may be due to reduced access to medical care and/or modifying genetic factors that influence the clinical phenotype. The incidence of all forms of ARA combined is approximately 5 in 10^7, although for the individual genes the number is considerably lower, with μ heavy chain gene mutations forming the largest group (Lopez-Granados et al., 2002), with a suggested incidence of 2.5 in 10^7. The various forms of ARA have been reported in all populations, but they occur more frequently in populations with a high index of consanguinity.

ONSET OF SYMPTOMS AND AGE AT DIAGNOSIS

In both XLA and ARA the onset of symptoms, mainly manifested as respiratory and/or gastrointestinal tract infections, is normally within the first year of life, after the disappearance of maternal IgG transported across the placenta. Among the 175 patients with XLA analyzed by Winkelstein et al. (2006), about 50 percent had symptoms during the first year. In a British study, which included 44 patients with XLA, 60 percent of affected boys had symptoms during their first year of life and 20 percent remained asymptomatic until the age of 3 to 5 years (Hermaszewski and Webster, 1993). In an Italian study of 73 patients with mutation-verified XLA, the mean age at onset of symptoms was 2 years (Plebani et al., 2002). The typical sites of infections in XLA are presented in Table 25.1. In reports from recent years there seems to be a tendency toward later onset of symptoms. This phenomenon

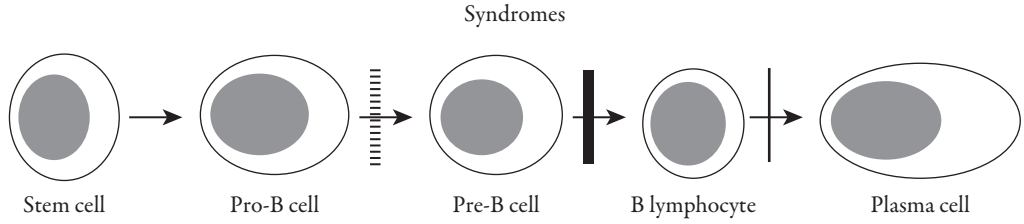

Figure 25.1 Schematic representation of B-lymphocyte development and the differentiation block/growth arrest in XLA. The broken line represents a partial block and the filled line an almost total block in B-lineage differentiation in XLA (modified from Mattsson et al., 1996). The thin broken line demonstrates the second block affecting B-cell differentiation as detected in mice with a targeted deletion of the *Btk* gene (Hendriks et al., 1996).

Table 25.1 INFECTIONS IN PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA

	PATIENTS IN EACH STUDY (%)				
PRESENTING INFECTION	LEDERMAN AND WINKELSTEIN (1985) *(N = 96)*	HANSEL et al. (1987) *(N = 69)*	HERMASZEWSKI AND WEBSTER (1993) *(N = 44)*	PLEBANI et al. (2002) *(N = 73)*	WINKELSTEIN et al. (2006) *(N = 201)*
Ear, nose, and throat	75	22	52	50	70
Pneumonia	56	67	32	39	62
Gastrointestinal infection	35	16	11	13	23
Bacterial skin infection	28	DC*	14	27	36
Meningitis	10	17	5	4	12
Septicemia	10	dc	7	6	10
Osteomyelitis	3	dc	5	—	3

*DC, different classification used, preventing direct comparison.

Modified from Sideras and Smith (1995).

may be caused by a generous contemporary usage of more efficient antibiotics.

Winkelstein et al. (2006) reported a mean age at diagnosis of 2.6 years for patients with a family history of immunodeficiency and 5.4 years for those without. In patients with mutation-proven sporadic XLA, diagnosed between 1990 and 2001, Conley and Howard found that the mean age at diagnosis was 35 months but the median age was 26 months. They noted that the diagnosis of XLA in a patient without a family history of disease was usually made when the patient was hospitalized for a severe or unusual infection.

Although the mean age at the onset of symptoms has increased, the mean age at diagnosis of XLA has decreased in all populations (Hansel et al., 1987; Plebani et al., 2002). Moin et al. (2004) reported that the age at diagnosis in the Iranian population had decreased considerably over the past 30 years. This decrease can be attributed to increased awareness of the disease but also the availability of mutation detection with resulting carrier detection and early genetic analysis in families with a history of XLA.

A subset of patients with mutation-verified XLA is not recognized to have immunodeficiency until adulthood (Conley and Howard, 2002; Conley et al., 2008; Hashimoto et al., 1999; Kornfeld et al., 1996; Morwood et al., 2004; Stewart et al., 2001; Vihinen et al., 1995, 1996b, 2001; Wood et al., 2001). In some cases this is because the patient did not have symptoms severe enough to elicit an evaluation; in others symptoms were present but not pursued. It is noteworthy that onset may also vary among individuals within the same family carrying the same mutation in the *BTK* gene (Bykowsky et al., 1996; Kornfeld et al., 1996; Saffran et al., 1994; Wedgewood and Ochs, 1980), demonstrating that other factors may influence this process.

In general, patients with all forms of ARA have the onset of symptoms and are recognized to have immunodeficiency at an earlier age than patients with XLA (Conley et al., 2005b; Lopez-Granados et al., 2002). This can be attributed, at least in part, to the fact that XLA is a leaky defect in B-cell differentiation (Conley et al., 1985; Nomura et al., 2000; Noordzij et al., 2002; Tedder et al., 1985), whereas the block in B-cell development is more severe in patients with ARA. The mean age at diagnosis in 16 patients with μ heavy chain deficiency was 11 months (Lopez-Granados et al., 2002). However, there are exceptions. In 1955 Waldenström et al. (Kulneff et al., 1955) described two brothers and a distant cousin with agammaglobulinemia who were born between 1945 and 1949. Two of these patients survived into adulthood despite what would be considered by current standards inadequate gammaglobulin replacement. Later studies showed that these patients had mutations in μ heavy chain (Lopez Granados et al., 2002).

SPECTRUM OF INFECTIONS

The types of infection seen in patients with agammaglobulinemia are influenced by whether the patient is receiving replacement Ig therapy, the age of the patient, and the types of exposure the patient experiences. Almost all patients with agammaglobulinemia have recurrent upper and/or respiratory problems, and many have gastrointestinal infections (Conley and Howard, 2002; Hermaszewski and Webster, 1993; Lederman and Winkelstein 1985; Ochs and Smith, 1996; Winkelstein et al., 2006). In a high proportion of patients, particularly patients without a family history of disease, an evaluation for immunodeficiency occurs when the patient is hospitalized for a severe, complicated, or prolonged infection (Conley and Howard, 2002). After the initiation of Ig therapy, acute, severe infections are much less common (Howard et al., 2006; Quartier et al., 1999). However, low-grade, chronic, insidious infections and complications of agammaglobulinemia may compromise the quality of life in affected patients. Although rare patients with agammaglobulinemia develop opportunistic infections, such as *Pneumocystis jirovecii* pneumonia (Alibrahim et al., 1998; Kanegane et al., 2009; Rao and Gelfand, 1983) or JCV-mediated leukoencephalopathy (Bezrodnik et al., 1998; Teramoto et al., 2003), the most typical infections are those caused by encapsulated bacteria and enteroviruses.

Bacterial Infections

Bacterial infections are the major complications in XLA and ARA as well as other antibody deficiencies (Hermaszewski and Webster, 1993; Lederman and Winkelstein, 1985; Ochs and Smith, 1996; Spicket et al., 1991; Stiehm et al., 1986; Winkelstein et al., 2006). The typical sites of infections are presented in Table 25.1. In a survey of patients with XLA, Lederman and Winkelstein (1985) reported a history of respiratory tract and/or gastrointestinal tract infections in 91 percent of patients at the time of diagnosis. Similar findings were obtained by Hermaszewski and Webster (1993).

The most frequently observed organisms seen in patients with agammaglobulinemia include *H. influenzae* and *S. pneumoniae*. These organisms may cause sepsis, meningitis, cellulitis, pneumonia, arthritis, otitis, sinusitis, and/or conjunctivitis. Since the availability of the conjugated *H. influenzae* vaccine in the late 1980s and early 1990s, serious infections with *H. influenzae* have almost disappeared in otherwise healthy children. Sepsis, meningitis, or cellulitis due to *H. influenzae* in a vaccinated child should elicit an evaluation for antibody deficiency. After the initiation of Ig therapy, the incidence of serious infections with *H. influenzae* and *S. pneumoniae* decreases, but the majority of patients continue to have problems with otitis, sinusitis, and/or conjunctivitis (Howard et al., 2006; Winkelstein et al., 2006).

Pseudomonas or staphylococcal sepsis is a relatively common presenting finding in patients with agammaglobulinemia. This association, first recognized as early as 1963 (Speirs et al., 1963), occurs more often in patients less than 1 year of age and almost exclusively in patients with neutropenia (Conley and Howard 2002; Farrar et al., 1996; Kanegane et al., 2005; Lopez-Granados et al., 2002). Neutropenia and sepsis are not usually seen in patients receiving adequate Ig therapy.

Infections with the nonsporulating gram-negative rod *Campylobacter jejuni* have been reported to cause sepsis, gastrointestinal disease, erysipelas-like skin lesions, pericarditis, and recurrent fever in patients with agammaglobulinemia (Kerstens et al., 1992; Rafi and Matz, 2002). The diarrhea may be of long duration, but in some cases patients are asymptomatic (Chusid et al., 1987; Hermaszewski and Webster, 1993; Melamed et al., 1983; Schuster et al., 1996; van der Meer et al., 1986). Excretion of *C. jejuni* in the stool, which in untreated nonimmunodeficient subjects normally persists for 2 to 3 weeks, may be prolonged.

Infection with fastidious organisms related to *C. jejuni,* such as *H. cinaedi* (previously known as *C. cinaedi*) or *Flexispira rapini*, has been reported in patients with XLA who are on therapeutic doses of Ig (Cuccherini et al., 2000; Gerrard et al., 2001; Simons et al., 2004, and personal observation). Patients often have the insidious onset of lower extremity swelling with woody induration and pigmentation in the affected areas. Thrombophlebitis and necrosing skin lesions can develop. Signs of infection are initially minimal with no fever and no warmth in the affected area. The erythrocyte sedimentation rate (ESR) is usually elevated and there is mild anemia of chronic disease. These organisms are very difficult to culture and require prolonged therapy, often with intravenous antibiotics.

Systemic *Ureaplasma urealyticum* infections or infections with other *Mycoplasma* species are also frequent and may primarily cause symptoms in the respiratory tract, joints, and urogenital tract (Gelfand, 1993; Hermaszewski and Webster, 1993; Lederman and Winkelstein, 1985; Roifman et al., 1986; Stuckey et al., 1978; Webster et al., 1978). These organisms are often difficult to identify, and the course of disease may be prolonged, with an insidious onset. Symptoms may be severe, with accompanying weight loss. Combined infections of *Mycoplasma* and other bacterial species may also contribute to disease severity. Urogenital tract infections can cause urethral strictures and result in epididymitis and prostatitis (Hermaszewski and Webster, 1993; Ochs and Smith, 1996).

Viral Infections

Most viral infections run a normal course in XLA and ARA (Conley et al., 2009; Janeway et al., 1953; Lederman and Winkelstein, 1985; Ochs and Smith, 1996). There is no evidence to suggest that the members of the herpes family, such as cytomegalovirus (CMV), varicella, or Epstein-Barr virus (EBV), cause unusual problems in patients with agammaglobulinemia. Patients with XLA who acquired hepatitis C from contaminated IVIG did not have an unusually severe course of disease, in contrast to many patients with CVID (Adams et al., 1997; Bjørø et al., 1994; Razvi et al., 2001). There may be a slight increase in the severity of some viral infections in the first few years of life; Conley and Howard (2002) found that a high percentage of patients with sporadic XLA had been hospitalized for a viral infection prior to diagnosis. However, these infections were not severe enough to elicit concern about immunodeficiency. The neutropenia with sepsis seen as presenting findings in patients with XLA or ARA may be due to a higher incidence of viral-induced bone marrow suppression in patients who lack natural antibodies. After the development of T-cell immunity, these infections are less troublesome. By contrast, infections with enteroviruses, including poliovirus, echoviruses, and coxsackievirus, can cause chronic meningoencephalitis and sometimes a dermatomyositis/fasciitis-like syndrome in a subset of patients with agammaglobulinemia (Lopez Granados, 2002; McKinney et al., 1987; Misbah et al., 1992; Wyatt, 1973).

The incidence of chronic enteroviral infection in the current era is markedly decreased compared to that seen 20 to 30 years ago. This is most likely due to our ability to provide normal concentrations of serum IgG with newer preparations of gammaglobulin. However, a few patients who have been treated with adequate doses of Ig have developed progressive encephalitis with documented enteroviral infection (Misbah et al., 1992; Quartier et al., 1999, 2000), and others have developed progressive neurological disease of unclear etiology (Ziegner et al., 2002) that may be caused by enterovirus. It is possible that these patients acquired the enteroviral infection before they were started on Ig therapy. Patients with immunodeficiency may harbor vaccine-associated poliovirus for many years before they develop symptoms (DeVries et al., 2011; MacLennan et al., 2004). The same may be true for echovirus, the most common cause of progressive enteroviral encephalitis in patients with agammaglobulinemia (McKinney et al., 1987).

The presentation of enteroviral encephalitis in patients with agammaglobulinemia is quite variable. Although some

patients have an acute onset of fever and headache, an insidious onset is more common. Patients may show behavior changes, declining cognitive skills, hearing loss, ataxia, paresthesias, or seizures (McKinney et al., 1987). Some but not all patients have an increased number of lymphocytes in the cerebral spinal fluid (CSF). The protein and glucose may be normal, but more often the protein is increased and the glucose is decreased. Early in the course of the disease imaging studies with magnetic resonance imaging (MRI) or computed tomography (CT) may be normal, but later studies show cerebral atrophy affecting both gray and white matter. PCR may identify enterovirus in CSF samples that are negative in viral cultures (Quartier et al., 2000; Rotbart et al., 1990); however, PCR may be negative, especially in patients being treated with adequate or high doses of Ig (personal observation).

Enteroviral infection may also present as a dermatomyositis-like syndrome associated with dissemination of enterovirus (McKinney et al., 1987; Thyss et al., 1990). This form of the disease is characterized by peripheral edema, erythematous rash, and evidence of inflammation in skin and muscle biopsy specimens. The infection in the central nervous system seems to occur prior to dissemination of the virus. Edema or myositis with fasciitis may be found as the only manifestation of viral dissemination. Enteroviral hepatitis resulting in hepatomegaly and an increase in liver enzymes such as alanine aminotransferase are frequently seen in affected patients, usually associated with erythematous rashes and fever.

Vaccine-associated polio can be the cause of paralysis and chronic progressive enteroviral encephalitis in patients with agammaglobulinemia; however, not all patients with agammaglobulinemia who receive live polio vaccine acquire symptomatic disease (Wyatt, 1973). During the period in which babies routinely received the live Sabin vaccine at 2, 4, 6, and 18 months of age, approximately 2 to 5 percent of patients with hypogammaglobulinemia showed clinical signs of disease. Wyatt estimated that this was similar to the percentage of otherwise healthy children who developed polio after exposure to wild-type virus. The factors that make some patients more vulnerable to enteroviral infection are unknown but are likely to include modifying genetic factors.

The clinical course of enteroviral encephalitis, including vaccine-induced polio, is quite variable. Some patients have rapidly progressive disease whereas others demonstrate a slow decline characterized by periodic exacerbations followed by periods of stable disease lasting as long as 20 years. At autopsy the histological appearance is sometimes consistent with that seen in an end-stage, severe encephalitic process of long duration (Fig 25.2). Some patients with polio, even wild-type polio, have spontaneous recovery. At least two adults with XLA had wild-type polio as children in the 1950s and were left with some residual weakness but recovered before they were recognized to have immunodeficiency (Conley and Howard, 2002; Sarpong et al., 2002).

Treatment for patients with chronic enteroviral encephalitis is inadequate. Most would agree that higher doses of therapeutic Ig are warranted in affected patients, but some patients progress despite this (Misbah et al., 1992). Intraventricular or intrathecal Ig has been used (Erlendsson et al., 1985; Johnson et al., 1985; Kondoh et al., 1987; Misbah et al., 1992; Quartier et al., 1999, 2000), but this therapy is associated with a high risk of bacterial contamination of the reservoir, and the evidence that it is effective is weak. Ribavirin, an antiviral drug, has been used but does not appear to clear the virus (MacLennan et al., 2004; Misbah et al., 1992; Thibaut et al., 2011). Some success has been reported with the use of an experimental antiviral drug, Pleconaril (Rotbart and Webster, 2001).

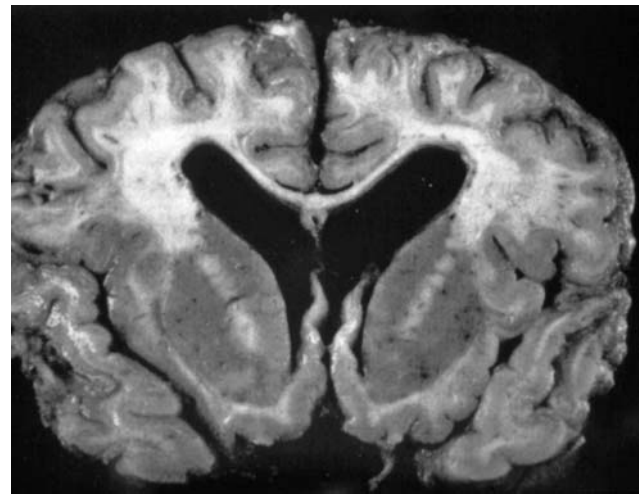

Figure 25.2 Whole-brain section of a patient with XLA having an enteroviral infection presumably contracted at 8 years of age. Autopsy performed at 17 years of age, after several years of progressing dementia, shows severe thinning of the cerebral cortex, reduced subcortical and deep white matter, and marked dilatation of the lateral ventricles. (Reproduced from Rudge et al., 1996).

CHRONIC AND RECURRENT DIARRHEA

Gastrointestinal infection caused by the flagellate *Giardia lamblia* has been found in many patients with XLA or ARA, although it appears to be more common in CVID (Hermaszewski and Webster, 1993; Lavilla et al., 1993; LoGalbo et al., 1982; Ochs et al., 1972). Winkelstein et al. (2006) reported that 12 of 46 XLA patients with chronic or recurrent diarrhea had a *G. lamblia* infection. In patients with XLA or ARA, giardia infection may not be associated with typical abdominal pain and diarrhea. Instead, malaise, fatigue, and an unexpectedly low concentration of serum IgG may be the early signs of infection. It is often very hard to eradicate giardia in these patients. Other causes of diarrhea in patients with agammaglobulinemia have included rotavirus, *Campylobacter fetus*, enterovirus, *Salmonella*, and cryptosporidia (Winkelstein et al., 2006, and personal observation).

ARTHRITIS

Arthritis was relatively common in patients with XLA in the 1960s and 1970s but is less common in the current era. Arthritis may be the presenting symptom and has been mistaken for chronic juvenile arthritis (Hansel et al., 1987). The arthritis is normally nonseptic and monoarthritic and affects the large joints, causing hydroarthritis with relatively little

pain (McLaughlin et al., 1972; Ochs and Smith, 1996). The arthritis frequently responds to gammaglobulin treatment (Plebani et al., 2002; Webster et al., 1976), and there is usually no apparent joint destruction. Residual IgG levels above 8 g/L were associated with improvement of clinical symptoms, whereas in patients with 5 to 8 g/L recurrences were seen (Quartier et al., 1999). The ESR is normal, as are the results of serological tests for rheumatoid factor and antinuclear antibodies. (Because these patients essentially lack immunoglobulins, autoantibody analysis is of no value.) Lederman and Winkelstein (1985) reported arthritis, including polyarthritis, in 20 percent of XLA patients. Less than half of the episodes were due to acute pyogenic infections. Conley and Howard (2002) reported pneumococcal arthritis in two patients diagnosed after 40 months of age. Enteroviruses or *Mycoplasma* species have also been identified in affected joints from some patients (Ackerson et al., 1987; Hermaszewski and Webster, 1993). An atypical case of rheumatoid arthritis with infiltrating CD8 cells and a case of psoriatic arthritis have been reported (Palazzi et al., 2003; Verbruggen et al., 2005).

AUTOIMMUNITY

In contrast to CVID (Cunningham-Rundles et al., 1987; Hermaszewski et al., 1991), XLA and ARA do not seem to predispose to most autoimmune disorders (Sideras and Smith, 1995). Type 1 diabetes has been reported in only a single patient with XLA, suggesting that humoral immunity is of great importance for the development of this form of autoimmunity (Martin et al., 2001). As mentioned in the section on arthritis above, cases of rheumatoid and psoriatic arthritis in XLA have also been reported (Palazzi et al., 2003;Verbruggen et al., 2005). However, inflammatory bowel disease (IBD) or a protein-losing enteropathy with features similar to IBD may be seen in 5 to 10 percent of patients with XLA or ARA (Cellier et al., 2000; Hermaszewski & Webster, 1993; Minegishi et al., 1999; Norman et al., 1975; Washington et al., 1996).

In some patients with agammaglobulinemia, classical signs of IBD may follow a documented infection with Giardia, enterovirus, or another pathogen. After the pathogen has been cleared, the patient may continue to have poor weight gain, abdominal pain, and low serum IgG despite adequate doses of therapeutic Ig. In other patients, no prior infection is identified. Aggressive immunosuppression, including high doses of steroids and an antimetabolite, is generally necessary to control the IBD. Concurrent therapy with antimicrobials may be helpful. Anti-TNF therapy does not appear to be useful in these patients.

ALLERGIC REACTIONS

Although one would expect patients with XLA or ARA to be free from typical allergies, patients occasionally develop seasonal allergies characterized by conjunctivitis and rhinorrhea; other patients have documented drug allergies. These patients have not had detectable serum IgE (personal observation). Kudva-Patel et al. (2002) described a patient with XLA who developed a diffuse, maculopapular rash with pruritus, hyperthermia, flushing, and lethargy after treatment with intravenous ceftriaxone; after drug challenge, the symptoms recurred. A skin biopsy showed the presence of infiltrating $CD4^+$, $CD45RO^+$ memory T cells in the absence of detectable IgE.

TUMORS

The question of whether there is an increased risk of malignancy in patients with XLA has not been resolved. Patients reported before 1993, when the gene responsible for XLA was identified, may have had one of the disorders that masquerades as XLA rather than XLA. Disorders associated with malignancy that can be similar to XLA include X-linked lymphoproliferative disease, CVID, and myelodysplasia.

There is some evidence for an increased incidence of gastrointestinal adenocarcinoma in patients with XLA. Van der Meer et al. (1993) described three patients with XLA and rectal adenocarcinoma. These patients, who were less than 40 years old, all had an extensive family history of XLA or a proven mutation in the *BTK* gene. Brosens et al. (2008) reported two cases of colonic adenocarcinoma in young men with mutations in *BTK*. Two young men with well-documented XLA and gastric carcinoma have been reported (Bachmeyer et al., 2000; Lavilla et al., 1993). We are aware of five additional XLA patients with adenocarcinoma of the gastrointestinal tract, all relatively young men. Some but not all of these men have had chronic gastrointestinal infection. Based on the observations that gastrointestinal adenocarcinoma is typically a malignancy of patients over 50 years of age, all of the reported XLA patients with this diagnosis have been less than 50 years old, and the relatively small number of patients with XLA who have reached adulthood, it is likely that there is an increased incidence of this cancer in patients with XLA. Adults with XLA, particularly those who have had chronic gastrointestinal infection, should be screened for colon cancer at a younger age than otherwise healthy individuals.

The basis of the increased risk of gastrointestinal cancer in patients with XLA is not clear. Chronic infection may lead to inflammation and dysplasia (van der Meer et al., 1993). There is likely to be altered gut flora in these patients due to their disease and antibiotic therapy. Glutathione-S-transferase activity has been found to significantly correlate with intestinal tumor incidence in humans, and decreased levels were identified in patients with XLA (Grubben et al., 2000). It is worth noting that human adenocarcinoma cell lines do not express BTK (Smith et al., 1994a).

A few XLA patients with other malignancies have been reported. Two patients with pituitary adenoma have been identified (Hermaszewski and Webster, 1993; Ochs and Smith, 1996). Von Recklinghausen disease was diagnosed at the age of 33 in one patient (Hirata et al., 2002). T-cell lymphomas were observed in two patients (Howard et al., 2006; Kanavaros et al., 2001). Squamous lung cancer has been reported in a 32-year-old patient with XLA (Echave-Sustaeta et al., 2001), and one patient with XLA has been diagnosed with osteosarcoma (personal observation).

No malignancies have been reported in ARA; however, the total number of patients identified is relatively small, and few of those patients have reached adulthood. If the malignancies in XLA are due to chronic inflammation, one would expect the incidence of tumors to be similar or greater in patients with ARA.

BTK has been implicated as tumor-inducing through an effect dependent on different splice variants induced by the fusion protein Bcr-Abl (Feldhahn et al., 2005a, 2005b). However, while the SH3 domain of BTK may serve as a substrate for the Abl cytoplasmic tyrosine kinase (Bäckesjö et al., 2002), a recent study found no evidence for a connection to tumors (MacPartlin et al., 2008).

Initial studies of patients with various forms of B-lymphoid tumors did not indicate any abnormal expression of BTK (Katz et al., 1994; Vorechovsky et al., 1994b). Furthermore, an increased frequency of chromosomal aberrations in the Xq22 region (in which the *BTK* gene is located) has to date not been reported in B-lineage tumors.

CAUSES OF DEATH

The causes of death in patients with XLA have changed over the years. Family studies suggest that patients who were born before 1960 often died suddenly at less than 5 years of age, most likely due to an acute bacterial infection. Although this still occurs, particularly in patients who are not yet receiving Ig replacement (Conley and Howard, 2002; Kanegane et al., 2005), it is uncommon in the current era. In surveys published before 1996 that included 170 patients with XLA, a total of 29 deaths were reported (Hermaszewski and Webster, 1993; Lederman and Winkelstein, 1985; Ochs and Smith, 1996). Twelve of these deaths were attributed to enteroviral infection, most often echovirus but also including coxsackievirus and vaccine-associated poliovirus. Chronic pulmonary disease was responsible for another nine deaths. Three patients died of hepatitis and the remaining patients died of other infections or inflammatory disease. Fatal adenocarcinoma of the gastrointestinal tract has been reported in patients with XLA (Bachmeyer et al., 2000; van der Meer, 1993).

In a more recent study that included 201 patients with XLA, Winkelstein et al. (2006) reported 17 deaths, 10 of which occurred after 1995. These more recent deaths included three patients with echovirus encephalitis and another young patient who died of myocarditis, perhaps due to coxsackievirus. Two patients died of chronic lung disease. Renal failure, hepatitis, adenoviral encephalitis, and complications of stem cell transplant were each responsible for one death. The causes of death were more diverse in a group of 10 adults reported by Howard et al. (2006). Only a single patient died of enteroviral encephalitis, and two died of chronic lung disease. Liver failure was the cause of death in four patients, three of whom had documented hepatitis C infection. The hepatitis C was acquired in the mid-1980s when several preparations of IVIG were contaminated with the virus. Although some patients with XLA or ARA may still have disease related to that contamination, one would expect the incidence of hepatitis C to decrease. One patient in that group died of T-cell lymphoma, one patient with inflammatory bowel disease died of a perforated bowel, and one died of a recreational drug overdose. As a higher proportion of patients with XLA are surviving into middle age and beyond, one would expect a greater proportion of patients with XLA to die of causes unrelated to their immunodeficiency.

Patients with ARA have died of enteroviral encephalitis and sepsis (Lopez-Granados et al., 2002, and personal observation). However, like the patients with XLA, many of those who were recognized to have immunodeficiency at an early age and who have been compliant with medical care have not had major problems.

LABORATORY FINDINGS

Patients with XLA and ARA generally have normal complete blood counts and serum chemistries. However, as noted above, profound neutropenia can be seen in patients who have not yet been started on adequate gammaglobulin replacement therapy (Buckley and Rowlands, 1973; Conley and Howard, 2002; Farrar et al., 1996). This neutropenia is unlikely to be caused by the absence of BTK in the myeloid lineage because neutropenia at presentation is more common in patients with ARA compared to those with XLA (Conley et al., 2005a), and most of the genes responsible for ARA are expressed exclusively in B-lineage cells. A low globulin fraction with normal serum albumin might alert the physician to the diagnosis of agammaglobulinemia.

The concentration of all serum Ig isotypes is markedly decreased in patients with agammaglobulinemia and the serum IgM and IgA are usually undetectable by a clinical laboratory (Table 25.2). Most patients with XLA have a small amount of measurable IgG and some patients have near-normal concentrations of IgG (Bykowsky et al., 1996; Conley and Puck, 1988; Plebani et al., 2002; Saffran et al., 1994, Wood et al., 2001). The serum IgM may be at the lower range of normal before gammaglobulin therapy is started, but it often decreases to less than 20 mg/dL after the initiation of treatment. Antibody to vaccine antigens is generally absent in patients with XLA and ARA, but there are rare exceptions (Conley et al., 2008; Wood et al., 2001).

The most characteristic laboratory finding in XLA and ARA is the marked reduction in the number and percentage of B cells (Table 25.2) in the peripheral circulation (Conley, 1985; Conley et al., 2005a; Cooper and Lawton, 1972; Naor et al., 1969; Sideras and Smith, 1995; Siegal et al., 1972; Yel et al., 1996). Most patients with XLA, particularly patients who are less than 10 years old, have a very small number of B cells in the blood (<1 percent of the total lymphocytes). These B cells have a distinctive phenotype characterized by increased expression of surface IgM and decreased expression of CD19 (Conley 1985; Dobbs et al., 2007). Most of these cells can be brightly stained for CD38 and CD24, which marks them as transitional B cells, B cells that have recently emigrated from the bone marrow (Cuss et al., 2006; Suryani et al., 2010).

Patients with XLA, ARA, or CVID with low numbers of B cells have reduced T-memory cells as detected by CD45RO

Table 25.2 CHARACTERISTIC LABORATORY FINDINGS IN PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA

SERUM IG LEVELS*	IGM	IGG	IGA
(g/L)	<0.1	<2	Often undetectable
Isohemagglutinins†	Undetectable		
Antibody responses to foreign antigens†	Undetectable or markedly reduced		
Peripheral blood‡	CD19	CD20	Surface Ig+
B-lymphocyte markers	<2%	<2%	<2%

*Normal values vary somewhat among different laboratories but are typically as follows: IgM: 0.5–2 g/L; IgG, 7–16 g/L; IgA: 0.5–3 g/L in adults. Normal values for IgA may vary extensively until adolescence. The IgG level is typically >4 g/L at all ages. In the newborn it is similar to what is seen in adults, as a consequence of placental transfer; it will thereafter decrease until about 6 months of age.

†Analysis is not obligatory for diagnosis, but it is recommended in patients having essentially normal Ig levels. For definitive diagnosis of XLA, mutation analysis has to be carried out.

‡Normal values vary somewhat among different laboratories, but are typically 5 to 20 percent, corresponding to $0.08–0.4 \cdot 10^9$ cells/L. As surface Ig determination may be influenced by Fc receptor binding, the inclusion of CD marker analysis is recommended.

(Martini et al., 2011); however, it is not clear that this has an effect on T-cell function. There are reports indicating that antigen-specific T memory is normal in XLA (Paroli et al., 2002; Plebani et al., 1997), but there are also studies indicating that T-cell memory to some antigens may be impaired (Morales-Aza et al., 2009).

B cells make up the major cell type in germinal follicles, and germinal follicles constitute much of the bulk of lymph nodes. In patients with ARA or XLA lymph nodes, tonsils and adenoids are very small and germinal follicles are absent on histological analysis (Good, 1954; Sideras and Smith, 1995). In the lamina propria of the gut, plasma cells are typically absent (Ament et al., 1973; Buckley and Rowlands, 1973; Ochs et al., 1972; Pan et al., 2002).

Analysis of bone marrow from patients with XLA or one of the known forms of ARA has revealed a maturation block between terminal deoxynucleotidyl transferase (TdT) Cμ-negative pro-B-cells and Cμ-positive pre-B-cells (Campana et al., 1990; Nomura et al., 2000; Noordzij et al., 2002a). The severity of the block in XLA is variable, and a few pre-B-cells are seen in most patients. The proliferative activity of these pre-B-cells is impaired (Campana et al., 1990; Noordzij et al., 2002a; Pearl et al., 1978). The block in B-cell differentiation is more severe in most patients with ARA (Ferrari et al., 2007; Minegishi et al., 1998, 1999; Yel et al., 1996).

MOLECULAR BASIS OF X-LINKED AGAMMAGLOBULINEMIA

THE GENE DEFECTIVE IN X-LINKED AGAMMAGLOBULINEMIA ENCODES A TYROSINE KINASE

The molecular basis of XLA was identified using two different modes of gene cloning: positional cloning by applying cDNA selection on yeast artificial chromosomes from the implicated region, and investigation of novel protein tyrosine kinases (PTKs) expressed in B lymphocytes (Tsukada et al., 1993; Vetrie et al., 1993). These studies demonstrated that a gene, *BTK*, encoding a novel cytoplasmic PTK was defective in XLA. PTKs are enzymes that catalyze the phosphorylation of tyrosine residues in proteins with ATP as phosphate donor and belong to the enzyme classification (EC) group EC 2.7.1.112. PTKs are subdivided into receptor PTKs, which are membrane-spanning proteins such as the receptor for platelet-derived growth factor (PDGF), and cytoplasmic or nonreceptor PTKs, exemplified by the cellular homolog of Rous sarcoma virus, c-Src (Manning et al., 2002). In humans there are 58 receptor and 32 nonreceptor PTKs.

When the *BTK* gene was isolated, it was the first time a cytoplasmic PTK was implicated in a hereditary human disease. Subsequently, mutations in the genes encoding ITK, JAK3, and ZAP-70 cytoplasmic kinases have been shown to cause primary immunodeficiency (see Chapters 1, 10, and 15, respectively).

BTK is composed of 659 amino acids with a total molecular weight of 77 kDa; the corresponding mRNA is normally found as a single species of 2.7 kb (Smith et al., 1994b; Tsukada et al., 1993; Vetrie et al., 1993). BTK carries three domains, which are also found in SRC. These are from the C-terminus, the kinase domain of about 280 amino acids, also referred to as *SRC homology 1* (SH1); a region of about 100 residues, which binds to phosphorylated tyrosine residues, designated SH2; and a domain of about 65 amino acids known to interact with proline-rich stretches, referred to as SH3 (Pawson, 1995).

In addition, BTK contains two regions in the N-terminus, designated *Pleckstrin homology* (PH), with 140 amino acids, and *Tec homology* (TH), containing approximately 80 residues. The PH domain is found in more than 100 proteins and is believed to have a membrane-targeting function (Rameh et al., 1997; Salim et al., 1996). The TH region is composed

of an extension of the PH domain, designated the *Btk motif* (27 residues), and a proline-rich stretch (Smith et al., 1994b; Vihinen et al., 1994b). The Btk motif in the TH region has been shown to bind a Zn^{2+} ion (Vihinen et al., 1997b). This region was named after TEC, which was the first member identified in the family of PTKs to which BTK belongs (Mano et al., 1990). The other family members are ITK (also called TSK and EMT) and BMX (also called ETK). The kinase TXK (also RLK) lacks the PH domain but is related to the TEC family of kinases.

Mutations in ITK have recently been reported in five members of two different families (Huck et al., 2009; Stepensky et al., 2011). All five patients developed severe immune dysregulation with B-cell proliferation or Hodgkin's lymphoma after EBV infection. Disease-causing, loss-of-function mutations have not been reported for any of the other TEC family members to date. For comprehensive reviews of the TEC family see Hussain et al., 2011; Smith et al., 2001, and Takesono et al., 2002.

CHROMOSOMAL REGION CONTAINING THE *BTK* GENE AND DELETIONS EXTENDING INTO THE *TIMM8A*, *TAF7L*, AND *DRP2* GENES

The human *BTK* gene is located on the long arm of the X chromosome in a region designated Xq22.1 (Fig. 25.3). It is transcribed in a telomere (→) centromere direction (Vorechovsky et al., 1994a) and encompasses 37.5 kb (Hagemann et al., 1994; Ohta et al., 1994; Rohrer et al., 1994; Sideras et al., 1994), as depicted in Figure 25.3. Use of cDNA selection technology enabled identification of several RNA species expressed from this region in B cells and in B-cell progenitors (Vorechovsky et al., 1994a). A deletion extending into the closest flanking gene, the centromerically located gene initially referred to as *DXS1274E*, was found in a family with XLA and sensorineural deafness (Vorechovsky et al., 1994a). Mutations in this gene, later renamed *DDP* (deafness dystonia protein) and subsequently officially renamed *TIMM8A* (Translocase of inner mitochondrial membrane 8 homolog A), because it encodes a conserved mitochondrial protein, were found to cause Mohr-Tranebjaerg syndrome (Jin et al., 1996). Subsequently, patients with various deletions extending from the *BTK* gene into the *DDP* gene have been reported (Richter et al., 2001; Sediva et al., 2007). Moreover, two families have been described with deletions encompassing four genes, *BTK*, *TIMM8A*, *TAF7L*, and *DRP2* (Jyonouchi et al., 2007; Sediva et al., 2007). The phenotype of the extended deletion does not differ from patients with BTK and TIMM8A deficiency; however, it is likely that spermatogenesis is impaired in patients with TAF7L deficiency. A map of this chromosomal region is depicted in Figure 25.3.

GENOMIC ORGANIZATION OF THE *BTK* GENE

BTK was the first member of the TEC family for which the genomic organization was established. The human *BTK* gene comprises 19 exons, including a 5′ untranslated region (exon 1) and a 3′ exon containing both the C-terminal coding and a 3′ untranslated part (Hagemann et al., 1994; Rohrer et al., 1994; Sideras et al., 1994), as depicted in Figure 25.3. The size of the exons varies from 55 to 560 bp, and the introns range from 164 bp to 9 kb. In the mouse, a similar genomic organization was observed (Sideras et al., 1994), the difference being mainly an increased size of introns 4 and 12. The number of exons is identical in humans and mice, as are the exon–intron boundaries. Furthermore, the human, mouse, and rat BTK proteins are highly conserved, being 98 to 99 percent identical at the amino acid level (Lindvall et al., 2005; Sideras et al., 1994).

The promoter region of the *BTK* gene was initially inferred from sequence motifs in the DNA stretch 5' of the start site (Sideras et al., 1994). Through functional analysis of this region, binding sites for members of four families of transcription factors have been identified, Sp1/3, Spi-B/PU.1, and OCT1/OBF1 and nuclear factor (NF)-κB, all of which have been shown to bind and activate transcription (Himmelmann et al., 1996; Müller et al., 1996, 1999; Yu et al., 2008). Tissue specificity seems to be provided by the Spi-1/PU. 1 factors, which are selectively expressed among hematopoietic cells. BTK is expressed in all hematopoietic cells with the exception of T cells and plasma cells. This indicates that the *BTK* gene is turned on early during differentiation, and of note is that BTK expression has been observed in cells carrying the early progenitor marker CD34 (Smith et al., 1994a).

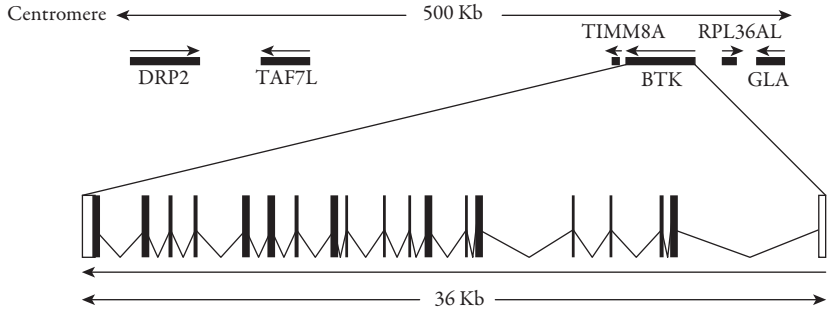

Figure 25.3 Physical map of the Xq22.1 region showing the *BTK* gene and adjacent genes (top) and how they are transcribed. Deletions encompassing the 3′ end of the *BTK* gene and extending into the *TIMM8A* gene cause XLA and Mohr-Tranebjaerg syndrome. Below is the organization of the *BTK* gene, showing the 19 exons. Filled squares represent translated exonic regions.

FUNCTIONAL ASPECTS OF PRE-BCR AND BCR AND OTHER FORMS OF RECEPTOR SIGNALING

MULTIPLE RECEPTORS CONNECT TO BTK IN B AND NON-B HEMATOPOIETIC CELL TYPES

During the development of the B-cell lineage immunoglobulin genes are rearranged, first giving rise to an expressed μ heavy chain, which together with VpreB and λ5 makes up the pre-B-cell receptor. Later, upon the successful generation of a light chain, the BCR is formed (reviewed in Kurosaki et al., 2010). None of these receptors carries an intracellular signaling domain, but instead they make use of accessory transmembrane signaling partners, namely Igα (CD79a) and Igβ (CD79b). Initial steps downstream of BCR activation include tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) sequences on Igα and Igβ by SRC family kinases. When the BCR gets activated, but also under "tonic signaling" (Monroe, 2006), a signalosome is formed containing BLNK (also known as SLP-65 or BASH), phospholipase Cγ2 (PLCγ2) and protein kinase C (PKC). Similarly, the pre-BCR generates a signaling complex (Guo et al., 2000). BTK's main role is to phosphorylate PLCγ2 on two tyrosine residues, resulting in the activation of this enzyme (Humphries et al., 2004). This generates inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). This in turn activates PKCβ in B lymphocytes and induces the release of Ca^{2+}, with subsequent activation of several downstream components, including NF-κB and other transcription factors. PKC plays a dual role by enhancing the downstream signaling while at the same time phosphorylating BTK, thereby inhibiting its activity (Yao et al., 1994). Another enzyme negatively regulating BTK through phosphorylated serines is the isomerase Pin1, which recognizes two separate serine residues in the PH (pleckstrin homology) domain (Yu et al., 2006). IP3 is formed after cleavage of phosphatidylinositol 3,4,5-trisphosphate (PIP3), and PIP3 is generated by phosphatidylinositol 3-kinase (PI3K). PIP3 is also of importance for BTK membrane tethering because the PH domain of BTK specifically binds to PIP3 (Rameh et al., 1997; Salim et al., 1996).

Apart from the pre-BCR and the BCR, many other receptors also activate BTK (Nore et al., 2000). One common denominator for many of these receptors may be PI3K. A variety of cytoplasmic molecules have been linked to BTK by physical or genetic means (reviewed in Mohamed et al., 2009).

ACTIVATION OF BTK BY RECEPTORS IN NON-B CELLS

BTK is broadly expressed among hematopoietic cell types, with the exception of T cells (Smith et al., 1994a). Important receptors, such as the Fcε receptor of mast cells, which mediates degranulation responses via IgE and allergen complex-induced binding, have been linked to BTK activation by genetic and biochemical criteria (Hata et al., 1998a; Kawakami et al., 1994). A different type of receptor activation is exemplified by the collagen-mediated activation of platelet aggregation via non-integrin glycoprotein receptors. Although XLA patients show no overt defect in blood clotting, BTK is clearly activated by this mechanism and may play an additive role with other Tec family kinases in mediating effective platelet activation (Quek et al., 1998). Toll-like receptor signaling has also been investigated in XLA non-B cells, but without any clinical implications (Marron et al., 2010; Sochorová et al., 2007).

ROLE OF BTK IN THE NUCLEUS

While BTK mainly resides in the cytoplasm, a potentially important BTK signaling event is to influence processes within the nucleus through the activation of transcriptional regulators. Evidence for BTK directly modifying transcription factors such as STAT5 (Mahajan et al., 2001) and TFII-I (Egloff and Desiderio, 2001; Novina et al., 1999) by tyrosine phosphorylation is one mode of control. The NF-κB transcription factor pathway has also been connected to BTK. BCR-induced activation of IKK and subsequent degradation of I-κB and activation of the NF-κB pathway is impaired in B cells lacking BTK, BLNK, or PLCγ2 (Bajpai et al., 2000; Petro et al., 2000; Petro and Khan, 2001; Tan et al., 2001). Interestingly, NF-κB also positively regulates the *BTK* promoter yielding a positive feedback loop (Yu et al., 2008). BTK was also found to shuttle between the cytoplasm and the nucleus (Mohamed et al., 2000). At least one transcription factor, Bright, is reported to bind directly to BTK (Webb et al., 2000) and relocalize as a complex within the nucleus. Bright also binds to lipid rafts (Schmidt et al., 2009). Recently an anchyrin-repeat-containing protein was found to regulate BTK shuttling by interacting with its SH3 domain (Gustafsson et al., 2012).

MUTATION ANALYSIS FOR *BTK*

BTK gene mutation detection is increasingly dependent on direct sequencing of exonic regions and splice sites, while previously most of the techniques used to identify mutations in BTK relied on PCR amplification, followed by a screening assay. Those were single-strand conformation polymorphism (SSCP) (Conley et al., 2004, Holinski-Feder et al., 1998; Vorechovsky et al., 1995a) or denaturing high-performance liquid chromotography (DHPLC) (Xiao and Oefner, 2001). Over 90 percent of mutations in BTK consist of single base pair substitutions or the loss or gain of less than 10 base pairs. The remaining mutations are more complex mutations, or deletions, insertions, or inversions (Conley et al., 2005b). Splice-site mutations are rather frequent. In rare cases, analysis of mRNA is needed to identify splice-site mutations because they occur in intronic regions far away from the regular consensus sites (Kralovicova et al., 2011).

In 1994 an international study group for XLA was formed with the aim of collecting all mutations into a common database (Vihinen et al., 1995). This database, designated BTKbase, is continuously updated and available through the Internet (http://bioinf.uta.fi/base_root/) (Väliaho et al., 2006). Investigators carrying out mutation analyses are

encouraged to contact the study group and report new information. Instructions are available at the BTKbase website or through one of the authors (C.I.E.S.). For further information on immunodeficiency disease mutation databases, including instructions for data submission, see Chapter 58.

The most recent edition of BTKbase, Version 8.53, last updated June 17, 2013 contains 1,254 public entries. Thus, the majority of affected families have mutations that are unique to their family. This observation can be explained, at least in part, by the fact that XLA is maintained in the population by new mutations. The high homology between human and murine BTK also suggests that very few alterations in the sequence are tolerated. No single mutation in the *BTK* gene accounts for more than 3 percent of patients (Conley et al., 2005a), and these represent CpG sites frequently affecting arginine codons (Väliaho et al., 2006).

Mutations in BTK are scattered throughout the gene, affecting every domain of the protein. Approximately 35 percent of mutations are single base pair substitutions resulting in amino acid substitutions. These missense mutations are not uniformly distributed but are preferentially found in the kinase domain, especially in the C-terminal portion of the kinase domain. A number of missense mutations also exist in the PH and SH2 domains, whereas the TH-SH3 region is much less affected (Lindvall et al., 2005).

Premature stop codons account for about 20 percent of families and single base pair substitutions resulting in splice defects are seen in an additional 15 to 20 percent of families. The insertion or deletion of 1 to 10 base pairs causes a frameshift mutation and a secondary premature stop codon in another 15 to 20 percent of families. A small number of in-frame deletions of 3 base pairs have been reported (Conley et al., 2005a; Väliaho et al., 2006). The premature stop codons, splice defects, and frameshift mutations are evenly distributed over the gene. A single mutation confined to the promoter region has been found (Holinski-Feder et al., 1998), but this type of mutation is very rare, typically corresponding to a few percent or less in *BTK* and other genes (Giannelli et al., 1996).

Certain DNA sequences are more vulnerable to mutations. For example, CpG sites are highly prone to mutagenic events. The 5-methylcytosine base frequently spontaneously deaminate to thymine, causing transitions (Duncan and Miller, 1980). These sites are also the major mutational hot spots for the sex-linked primary immunodeficiencies, including XLA (Conley et al., 2005a; Lindvall et al., 2005; Smith and Vihinen, 1996; Väliaho et al., 2006). Not all CpG sites in BTK are associated with disease, suggesting that selected sites might not be sensitive to replacement (Lindvall et al., 2005) or the DNA/chromatin structure may provide some protection to some sites.

Large deletions in the *BTK* gene, ranging in size from 2 kb to nearly 200 kb, are seen in about 5 percent of families with XLA. Some of these deletions extend into neighboring genes, including *TIMM8A*, *TAF7L*, and *DRP2*, as noted above (Richter et al., 2001; Rohrer et al., 1999; Sediva et al., 2007; van Zelm et al., 2008; Vetrie et al., 1993; Vorechovsky et al., 1994a). Deletions that include the 3' part of BTK almost invariably delete the gene *TIMM8A*, which is located less than 1 kb downstream of exon 19 of *BTK*. Of note, in some patients with XLA and deletions that include TIMM8A, deafness was initially attributed to chronic otitis or the use of toxic antibiotics. A small number of duplications of 2 or more exons in *BTK* and an inversion of at least 48 kb that disrupted *BTK* between exons 4 and 5 have been reported (Rohrer et al., 1999). Two unrelated patients with XLA have been shown to have two different retrotransposon insertions at exactly the same site immediately downstream of exon 9 of *BTK* (Conley et al., 2005b).

Initial studies suggested that there was no correlation between the specific mutation in *BTK* and the severity of disease (Holinski-Feder et al., 1998; Vihinen et al., 1996a). However, analysis of larger numbers of patients indicates that some mutations are more likely to result in an older age at diagnosis, a slightly higher concentration of serum immunoglobulins, and a few more B cells in the peripheral blood (Broides et al., 2006; Lopez-Granados et al., 2005; Noordzij et al., 2002b; Plebani et al., 2002). Amino acid substitutions, particularly amino acid substitutions that allow the production of some BTK protein, and splice defects that occur at sites in the splice consensus sequence that are conserved but not invariant are more likely to be associated with a milder phenotype. The genotype–phenotype correlation is weak, and patients from the same family with the same mutations may have striking differences in clinical findings (Buckley and Sidbury, 1968; Bykowsky et al., 1996; Goldblum et al., 1974; Kornfeld et al., 1996; Saffran et al., 1994; Wedgwood and Ochs, 1980). This suggests that there are modifying genetic or environmental factors that influence the severity of disease.

MUTATION ANALYSIS FOR ARA

Mutations in μ heavy chain account for the largest proportion of patients with ARA. The μ heavy chain locus is complex with 50 to 120 VH regions, half of which are pseudogenes (Cook et al., 1994; Matsuda et al., 1993), approximately 30 DH segments (Ichihara et al., 1988; Schroeder et al., 1988), and 6 JH regions upstream of the μ constant region. There are polymorphic variations in the number of VH, DH, and JH segments. The search for mutations in μ heavy chain has focused on the 6 exons that encode the constant region (CH) of μ heavy chain. The first 4 exons encode the individual CH domains and the last 2 exons encode the sequences required to produce a membrane form of μ heavy chain.

Twenty-six families with mutations in the constant region of μ heavy chain have been identified (Ferrari et al., 2007b; Lopez-Granados et al., 2002; Meffre et al., 2001; van Zelm et al., 2008; Yel et al., 1996; Zhang et al., 2010, and unpublished observation). The spectrum of mutations in μ heavy chain differs quite remarkably from that seen in BTK. Half of the mutations are large deletions, 71 to 732 kb in length, that remove all of the constant region exons and usually additional segments of the μ heavy chain locus, including some VH segments, DH segments, JH segments, and other constant region genes (Lopez-Granados et al.,

2002; van Zelm et al., 2008; Yel et al., 1996, and unpublished observations). Eight frameshift mutations, four deletions, three insertions, and one complex combination of insertion and deletion have been reported. Only four single base pair substitutions have been reported: one resulting in a premature stop codon in exon 3, two causing amino acid substitutions in exon 4, and one occurring at the −1 position of the alternative splice site in exon 4 (Lopez-Granados et al., 2002).

In contrast to XLA, in which no single mutation in BTK accounts for more than 3 percent of patients, several mutations in μ heavy chain have been seen in multiple families. The most common mutation is the single base pair substitution at the alternative splice site. This G-to-A substitution, which occurs at a CpG site, has been identified in eight unrelated families from all over the world (Ferrari et al., 2007; Lopez-Granados et al., 2002; Zhang et al., 2010). The mutation has been seen on least three different μ heavy chain haplotypes (Lopez-Granados et al., 2002). By contrast, shared descent is clearly responsible for a 2 bp deletion at codon 168 in exon 2. This mutation has been seen in four unrelated families, two of whom are Spanish and two of whom are Hispanic-American. This mutation occurred on the same uncommon haplotype of the μ heavy chain locus (Lopez-Granados et al., 2002, and unpublished observation). Two Turkish families with the same 72 kb deletion have also been reported (van Zelm et al., 2008). At this time there does not appear to be a genotype–phenotype correlation in patients with mutations in μ heavy chain. All of the reported defects result in a complete block in B-cell differentiation at the stage when the pre-BCR should be expressed on the cell surface.

Mutations in other components of the pre-BCR or BCR may also result in profound hypogammaglobulinemia and markedly reduced or absent B cells. As noted above, the pre-BCR is composed of μ heavy chain, the transmembrane signal transduction molecules Igα and Igβ, and the surrogate light chain (Melchers, 2005). The surrogate light chain is made up of two B-cell specific proteins, VpreB and λ5/14.1 (Kudo and Melchers, 1987; Melchers et al., 1993; Sakaguchi and Melchers, 1986), encoded within the lambda light chain locus on chromosome 22q11.2 (Bauer et al., 1993; Bossy et al., 1991; Erikson et al., 1981). These two proteins assemble to produce a complex that is very similar to a lambda light chain. The amino-terminal portion of VpreB has high homology to the variable region of an immunoglobulin molecule and the carboxyterminal portion of λ5/14.1 has homology to the J region and constant region of lambda light chain (Kudo and Melchers, 1987; Sakaguchi and Melchers, 1986). They are noncovalently linked to each other (Kerr et al., 1989; Minegishi et al., 1999) and covalently linked to the μ heavy chain via a cysteine residue in the carboxyterminal portion of λ5/14.1 (Kerr et al., 1989; Pillai and Baltimore, 1987). Together, VpreB and λ5/14.1 form a surrogate light chain that escorts the rearranged μ heavy chain to the cell surface prior to the rearrangement of the conventional light chain genes. This allows the B-cell precursor to test the integrity of the rearranged μ heavy chain before investing in extensive proliferation or additional gene rearrangements.

The genes for VpreB and λ5/14.1 are relatively small—2 exons and 3 exons, respectively. Because there are two λ5 pseudogenes that have over 95 percent homology with exons 2 and 3 of λ5/14.1, care must be taken to develop assays that are specific for the functional gene (Minegishi et al., 1998). Although no mutations in VpreB have been reported, two patients with defects in λ5/14.1 have been identified (Conley et al., 2009; Minegishi et al., 1998). The first patient was recognized to have immunodeficiency at 3 years of age when he developed meningitis and arthritis due to *H. influenzae*. Panhypogammaglobulinemia, absent titers to vaccine antigens, and a lack of peripheral blood B cells were seen and he was started on IVIG. At later evaluations he has had serum IgM and IgA that were less than 8 mg/dL and less than 0.02 percent $CD19^+$ cells in the blood. Mutation studies documented a premature stop codon in exon 1 at codon 22 on the maternal allele and 3 base pair substitutions in exon 3 at codons 131, 140, and 142 on the paternal allele. The first two substitutions would not be expected to alter the coding sequence; however, the C-to-T transition at nucleotide 425 would be predicted to replace the invariant proline at codon 142 with a leucine. Protein folding studies demonstrated that this alteration resulted in an inability of the λ5/14.1 protein to fold properly (Minegishi et al., 1998).

Of interest, the 3 base pair substitutions in the paternal allele of λ5/14.1 are the same as those found at the corresponding site in exon 3 of the λ5/14.1 pseudogene 16.1, suggesting that this mutations was the result of a gene conversion event (Minegishi et al., 1998). Analysis of λ5/14.1 in controls showed that this gene is highly polymorphic. Thirteen variant alleles of λ5/14.1, the majority of which could be attributed to gene conversion events, were found in 134 unrelated individuals (Conley et al., 1999). Nine of these variants resulted in amino acid substitutions. Although gene conversion is an unusual mechanism of mutation, some species use gene conversion to generate diversity in the variable region of immunoglobulin and immunoglobulin-like loci (Bajoghli et al., 2011; Reynaud et al., 1987; Thompson 1992). Immunoglobulin and immunoglobulin-like genes may be unusually susceptible to gene conversion events.

The second patient with mutations in λ5/14.1 had the onset of recurrent otitis and sinusitis in early childhood and had his first pneumonia at 18 years of age. He was hospitalized for pneumococcal pneumonia and sepsis at 28 and 32 years of age. An immune evaluation documented a serum IgG of 330 mg/dL, IgM 12.5 mg/dL, and IgA 6 mg/dL. By flow cytometry, he had less than 0.01 percent $CD19^+$ cells. Mutation detection documented a homozygous single base pair deletion, a G deletion in codon 85 in exon 2 of λ5/14.1.

The signal transduction molecules Igα and Igβ (also called CD79a and CD79b or mb-1 and B29) are essential components of the pre-BCR and BCR complex (Hombach et al., 1990). Both molecules are B-cell-specific proteins with structural similarities to the CD3γ, δ, and ε chains in T cells. Both have a single extracellular immunoglobulin domain, a transmembrane domain, and an intracytoplasmic domain containing an ITAM characterized by the sequence $YXXL(X)_7YXXL$ in which Y represents tyrosine, L represents leucine, and X

denotes any amino acid. Igα and Igβ form a covalently linked heterodimer that masks the hydrophobic transmembrane domain of μ heavy chain and escorts it to the cell surface. When the BCR is cross-linked, an SRC family member phosphorylates the tyrosines in the ITAM motif, converting this sequence into a docking site for the downstream tyrosine kinase, SYK.

The gene for Igα consists of 5 exons encoded at 19q13.2 (Ha et al., 1994), and the gene for Igβ is made up of 6 exons at chromosome 17q23 (Wood et al., 1993). Four patients with defects in Igα have been identified (Conley et al., 2009; Minegishi et al., 1999; Wang et al., 2002). All have been homozygous for their mutations. The first, a Turkish girl, was found to have a A-to-G substitution at the invariant −2 position of the splice acceptor site for exon 3. This mutation resulted in the skipping of exon 3, which contains the region encoding the transmembrane domain of Igα (Minegishi et al., 1999). A different splice defect, a G-to-A substitution at the +1 position of the splice donor site for exon 2, was identified in another Turkish child (Wang et al., 2002). The third mutation, a premature stop codon due to a G-to-T substitution in codon 48 in exon 2 in Igα, was identified in a little girl from Mexico (Conley et al., 2009). The last patient, of Pakistani descent, had a 2 base pair deletion, a GC deletion at codon 68, exon 2 of Igα. All of these mutations would be expected to result in absence of Igα protein. All four patients were hospitalized for infection by 13 months of age, and two of the patients had clinical findings consistent with vaccine-associated polio or enteroviral-induced dermatomyositis. All of the patients had less than 1 percent $CD19^+$ cells. Two patients, the first and the third, were evaluated in more detail at 2 to 5 years of age. Both had less than 0.01 percent $CD19^+$ cells in the blood. Bone marrow studies documented a block in differentiation at the pro-B to pre-B cell transition that was indistinguishable from that seen in patients with null mutations in μ heavy chain (Minegishi et al., 1999).

Two patients with defects in Igβ have been identified. One patient, an Italian, was homozygous for a C-to-T single base pair substitution at codon 80, resulting in a premature stop codon in exon 2 (Ferrari et al., 2007b). The other patient, from the Caucasus Mountains, had a single base pair substitution in codon 137 (Dobbs et al., 2007). The change at this site, which is immediately downstream of the cysteine that forms the disulfide bridge with Igα, replaces the wild-type glycine with serine. This glycine is conserved not only in Igβ from humans, mice, dogs, and cattle but also in Igα from humans, mice, dogs, and cattle.

Both patients with mutations in Igβ were hospitalized for infection in the first year of life. The patient with a premature stop codon had laboratory findings that were the same as those seen in the patients with mutations in Igα. He had less than 1 percent $CD19^+$ cells in the peripheral circulation and a complete block in B-cell development at the pro-B to pre-B cell transition (Ferrari et al., 2007b). The patient with the G137S mutation in Igβ had a small number of B cells in the peripheral circulation (0.08 percent $CD19^+$ cells). These B cells showed striking similarities and differences when compared to those seen in patients with mutations in *BTK*. B cells from both were variable in intensity of CD19 expression and had increased expression of CD38 and decreased expression of CD21. However, the B cells from the patient with the Igβ mutation showed decreased or absent expression of surface IgM, whereas those from patients with mutations in *BTK* showed increased expression of surface IgM. These findings suggested that the alteration in Igβ influenced the ability of the BCR to reach the cell surface.

To examine the ability of the G137S mutant Igβ to bring the BCR to the cell surface, Jurkat T cells were transfected with retroviral vectors that would allow the production of a wild-type or mutant BCR (Dobbs et al., 2007). Cells transduced with the mutant Igβ consistently demonstrated less surface IgM in which all of the components of the BCR were wild type.

Cross-linking of the BCR complex initiates a cascade of tyrosine phosphorylation events. One of the first proteins to be phosphorylated is a 456-amino-acid adaptor protein referred to as BLNK (Fu and Chan, 1997; Fu et al., 1998; Goitsuka et al., 1998) (also called SLP-65 and BASH), which is encoded at 10q23.22 in 17 exons. BLNK has significant homology to SLP-76, a protein expressed in T cells, myeloid cells, and platelets (Clements et al., 1998; Jackman et al., 1995). Once BLNK is phosphorylated by Syk, it acts as a scaffold to assemble to the downstream targets of BCR-mediated activation, including BTK, PLCγ2, Grb2, Vav, and Nck.

Several observations suggested that mutations in the *BLNK* gene might result in a selective defect in B-cell development. First, expression of BLNK is limited to B cells and myeloid cells (Fu et al., 1998). Second, absence of BLNK in a chicken B-cell line, DT40, like absence of BTK, results in defective calcium mobilization (Ishiai et al., 1999; Kurosaki, 1999). Third, mice that are null for the T-cell homolog of BLNK, SLP-76, fail to develop T cells (Clements et al., 1998; Pivniouk et al., 1998).

Three patients with mutations in *BLNK* have been identified. The first, a 20-year-old male from Appalachia, had two noncontiguous base pair substitutions in exon 1. The first alteration, a C-to-A substitution at the third base pair in codon 10, does not change the proline encoded at this position and would not be expected to have functional consequences. The second alteration, an A-to-T substitution, was seen at the conserved +3 position of the splice donor site of intron 1, 20 base pairs downstream from the alteration in codon 10 (Minegishi et al., 1999). Analysis of cDNA from the bone marrow of this patient demonstrated a complete absence of *BLNK* transcripts.

The second patient, a Turkish girl, was homozygous for a C-to-T base pair substitution in codon 123, resulting in the replacement of the wild-type arginine with a premature stop codon (Conley et al., 2009). This patient had a very small number of B cells in the peripheral circulation, approximately 0.01 percent. By analyzing 500,000 events, it was possible to show that the phenotype of these cells was very similar to that seen in patients with mutations in the *BTK* gene. The cells demonstrated increased expression of surface IgM but variable and decreased expression of CD19. The shared phenotype of B cells from patients with mutations in *BTK* or *BLNK* suggests

that the decreased expression of CD19 is due to faulty signaling through the BCR.

TREATMENT

GAMMAGLOBULIN REPLACEMENT THERAPY

There is widespread agreement that Ig therapy, which is discussed in detail in Chapter 59, is essential for patients with antibody deficiencies, including patients with XLA or ARA (Cunningham-Rundles 2011; Orange et al., 2010; Roifman et al., 1985). Both intravenous and subcutaneous routes of administration can be used to provide normal concentrations of serum IgG. Intramuscular administration and plasma therapy have been used in the past but do not allow an adequate level of IgG to be attained. The exact dose of Ig that should be used may depend on the clinical situation. Patients with chronic lung disease or enteroviral infection may need a higher dose than patients who were started on Ig therapy at an early age and have minimal complications of the disease. In an industry-sponsored meta-analysis of clinical studies comparing various doses of gammaglobulin in 676 antibody-deficient patients, Orange et al. (2010) reported that pneumonia incidence declined by 27 percent for each 100-mg/dL increment in trough IgG. However, the differences were most noticeable for patients who had a trough level less than 600 mg/dL, and none of the studies stratified patients based on the presence of chronic lung disease prior to the onset of Ig therapy.

It seems likely that high doses of Ig may reduce the risk of enteroviral infection (Misbah et al., 1992; Quartier et al., 1999, 2001), although this has not been formally proven. Thus, the risk of enteroviral infection appears to be limited to patients with profound hypogammaglobulinemia and likely also to patients infected with rare forms of enteroviruses against which protecting antibodies in gammaglobulin preparations are scarce. A dose of 400 to 600 mg/kg/4 weeks, the standard dose of gammaglobulin, should result in an IgG trough of at least 600 mg/dL. If the serum IgG is lower than that, one should consider protein loss, particularly through the gastrointestinal tract.

There are no published studies that indicate superiority of of either intravenous or subcutaneous Ig therapy. Subcutaneous administration is less likely to result in side effects, and some patients find it more convenient. In a series of patients treated with high-dose subcutaneous γ-globulin, the majority of whom had CVID, only 0.2 patient days/year were spent in the hospital for respiratory tract infections (Gardulf et al., 1991). Furthermore, a significantly increased health-related function and improved self-rated health were reported (Gardulf et al., 1993, 1995). Subcutaneous delivery may also be in the form of express infusions, further decreasing the time (Hansen et al., 2002).

Like all drugs, Ig therapy can be associated with side effects or complications. In the 1980s hepatitis C-contaminated IVIG batches caused several fatal cases of infection worldwide in immunoglobulin-deficient patients (Bjøro et al., 1994; Razvi et al., 2001; Rossi et al., 1997). However, current procedures for gammaglobulin manufacturing are much less likely to transfer infectious agents of any kind. Although hepatitis C may run a more severe course in patients with agammaglobulinemia than in otherwise healthy individuals, patients with XLA and hepatitis C appear to develop a less fulminant hepatitis than that in CVID patients (Bjøro et al., 1994; Hermaszewski and Webster, 1993; J. Björkander, personal communication). In one series reported by Bjøro et al. (1994), all five infected CVID patients developed severe cirrhosis, including one with a fatal outcome, whereas only one of six XLA patients developed cirrhosis and end-stage liver disease. This patient was co-infected with hepatitis B and D viruses. However, in a study by Razvi et al. (2001), the outcome of a hepatitis C infection did not differ between CVID and XLA patients, whereas Quinti et al. (2002) reported a better prognosis in XLA than that in CVID.

Severe adverse events, including myocardial infarction, renal failure, and thromboembolitis, have been reported in adults treated with high doses of intravenous Ig for autoimmune disease, but these complications have not been seen in immunodeficient patients receiving standard Ig doses (Gupta et al., 2001; Rajabally and Kearney, 2011).

ANTIBIOTICS

Treatment of infections is essential in all forms of primary immunodeficiency. The use of antibiotics to combat bacterial infections represents one of the cornerstones in the management of agammaglobulinemia, and prolonged administration is often necessary. Some centers use chronic prophylactic antibiotics; others cycle antibiotics. The use of low-dose chronic antibiotics is not recommended because of the increased risk of developing bacterial resistance. Chronic prophylactic antibiotics seem to be more beneficial to patients who are less than 15 years old than in teenagers and adults, perhaps because of anatomical vulnerability or because T-cell immunity has not yet developed. If a patient develops a new cough that lasts more than 3 weeks or if a patient is hospitalized for infection, a course of antibiotics of at least 6 weeks should be given. However, as in CVID, it is frequently not possible to eradicate an infectious agent completely. For a more detailed description of treatment with antibiotics, see Chapter 59.

BONE MARROW TRANSPLANTATION AND GENE THERAPY

Bone marrow transplantation is an option for patients with agammaglobulinemia, as for other primary immune deficiencies. However, transplantation is not a risk-free procedure, as graft-versus-host reactions and severe infections may ensue. The stem cell grafting procedure may or may not be ablative. Thus, conditioning increases the chance for engraftment, whereas under nonablative conditions insufficient chimerism may develop. Howard et al. (2003) reported on three patients receiving cord blood or bone marrow grafts from human leukocyte antigen (HLA)-matched siblings without receiving any preparative regimen or antirejection drugs, and another three who received treatment with cyclosporin A and

mycophenolate mofetil after transplant. However, none of the patients showed an increase in serum IgM or peripheral B-cell counts.

Gene therapy was suggested as a future treatment in both the original reports on cloning of the *BTK* gene (Tsukada et al., 1993; Vetrie et al., 1993). In theory, the defect underlying XLA makes this disease particularly suitable for gene therapy, since the differentiation defect would enable gene-corrected cells to selectively proliferate and/or survive. This characteristic is seen also in adenosine deaminase (ADA) deficiency and severe combined immune deficiency caused by mutations in the common γ-chain (*XSCID1*), mainly affecting T-lineage development. For both these disorders, successful gene therapy has been conducted (see Chapter 61). Further, animal studies suggest that expression of 25 to 50 percent of the normal amount of BTK by homozygous transgene or retroviral vectors corrects serum immunoglobulin concentrations but not the ability to make antigen-specific antibodies (Conley et al., 2000; Satterthwaite et al., 1997). Because all forms of ARA are rare, it is less likely that techniques that are gene-specific will be developed for these disorders.

PROGNOSIS

Prior to antibiotic therapy and γ-globulin substitution therapy, the prognosis of patients with XLA or ARA was poor. Analysis of patients born before 1950 in a large Dutch pedigree revealed that there was a 90 percent probability of children with XLA dying between 2 months and 8 years of age (Mensink et al., 1984). With the advent of antibiotics and γ-globulin replacement therapy in the 1950s, the prognosis improved considerably (Lederman and Winkelstein, 1985), and it continues to improve (Hermaszewski and Webster, 1993; Howard et al., 2006; Ochs and Smith, 1996). This can be attributed to earlier diagnosis, more effective antibiotics, and the ability to achieve normal serum concentrations of IgG with newer preparations of γ-globulin. The majority of patients with XLA who were born after 1980 are reaching adulthood, and many of them have not been hospitalized for infection after initiation of therapy (Howard et al., 2006). The potential for gene therapy for XLA (Moreau et al., 2007; Ng et al., 2010; Sather et al., 2011; Yu et al., 2004) encourages us to think that further improvements in prognosis and quality of life will be seen in the future.

DIFFERENTIAL DIAGNOSIS

In a male child with the early onset of recurrent bacterial infections, panhypogammaglobulinemia, and less than 1 percent CD19+ cells, a mutation in the *BTK* gene can be identified in approximately 85 percent of patients (Conley et al., 1998). Some mutations affecting BTK, including duplications, inversions, and mutations that occur outside of the coding regions, are difficult to detect by standard techniques. If there is no BTK protein in monocytes or platelets (Futatani et al., 1998, 2001), additional techniques including Southern blot analysis and/or cDNA sequencing may be required to identify the mutation (Kralovicova et al., 2011, Rohrer et al., 1999).

A female with agammaglobulinemia and absent B cells is unlikely to have mutations in BTK. During embryogenesis of the female, random inactivation of one of the two X chromosomes occurs in every somatic cell and all of the descendants of that cell (Lyon, 1966). Thus, the normal female is a mosaic with approximately half of the cells in every tissue having the maternally derived X as the active X, and the remaining cells having the paternally derived X as the active X. However, if one of the two X chromosomes carries a gene defect that is detrimental to the proliferation or survival of cells of a particular lineage, all the cells of that lineage will have the nonmutant X chromosome as the active X. BTK provides a very strong selective advantage to B-cell precursors, such that a female heterozygous carrier of a *BTK* mutation has normal numbers of B cells, all of which were derived from B-cell precursors with the nonmutant X chromosome as the active X (Conley et al., 1986; Conley and Puck, 1988; Fearon et al., 1987). One can anticipate that a female might have XLA if she had Turner's syndrome (females with a single X chromosome and no Y chromosome), uniparental disomy (two copies of the same affected X chromosome), or one X chromosome bearing the BTK mutation and the other X unable to function as an active X chromosome (Conley and Sweinberg, 1992). The latter situation has been reported in a single female whose father had XLA and the maternally derived X could not be used as the active X chromosome (Takada et al., 2004). No other female patients with XLA have been reported.

Male and female patients who have an "XLA phenotype" but no mutation affecting BTK may have one of the autosomal recessive forms of agammaglobulinemia (Conley et al., 2009). ARA is more likely in consanguineous families or families who live in a geographically isolated area. About 75 percent of patients with ARA are homozygous for their mutation, indicating that their parents have an ancestor in common (Conley et al., 2005a). Screening of genomic DNA is the most reliable way to identify one of the autosomal recessive forms of agammaglobulinemia. A single patient with mild dysmorphic features, hypogammaglobulinemia, reduced numbers of peripheral B cells, and a translocation in the gene for LRRC8 has been reported (Sawada et al., 2003). This clinical syndrome may be due to the expression of a LRRC8-fusion protein rather than an abnormality in expression of LRRC8 itself.

Other immunodeficiencies or clinical disorders can be confused with XLA or ARA. A single family has been described in which the four affected males had hypogammaglobulinemia, decreased or absent B cells, and growth hormone deficiency inherited in an X-linked pattern (Fleisher et al., 1980). Screening of the *BTK* cDNA in this family did not reveal any mutations, and normal BTK protein was detected by Western blot (Stewart et al., 1995). Alterations in the *ELF4* gene in this family have been implicated, but the results are inconclusive (Stewart et al., 2008). Several other males with XLA and growth hormone deficiency have been reported, but mutations in BTK have been found in all of these patients (Conley et al., 1994; Duriez et al., 1994; Vihinen et al., 1994c).

Some patients with WHIM syndrome have hypogammaglobulinemia and reduced numbers of B cells (Hernandez et al., 2003; Kawai and Malech 2009). Although many of these patients, particularly young children, do not have a history of warts, most have persistent neutropenia. Some patients with XLP have reduced numbers of B cells as well as hypogammaglobulinemia (Morra et al., 2001). Patients with myelodysplasia, including the various forms of dyskeratosis congenital, may have hypogammaglobulinemia with markedly reduced numbers of B cells (Gupta and Kumar, 2010; Srivannaboon et al., 2001). These patients usually, but not always, have other hematopoietic abnormalities. Some patients with CVID have severely reduced numbers of B cells in the peripheral circulation (Cunningham-Rundles and Bodian, 1999; Wehr et al., 2008). In a patient with absent B cells, particularly a male patient, the diagnosis of XLA or one of the autosomal recessive forms of agammaglobulinemia should be considered before the patient is given the diagnosis of CVID. An early onset of recurrent infections and a family history of disease or consanguinity suggest that more detailed genetic analysis might be appropriate, irrespective of the patient's age. However, there are clearly patients who have delayed onset of infections, severe hypogammaglobulinemia, and absent B cells who do not have mutations in any of the known genes associated with agammaglobulinemia.

ANIMAL MODELS OF BTK LOSS OF FUNCTION

Shortly after *BTK* was identified as the defective gene in XLA, two groups showed that a well-characterized mouse model of immunodeficiency, X-linked immunodeficiency or xid mice, is caused by a point mutation in the PH domain of BTK that leaves kinase activity intact (Rawlings et al., 1993; Thomas et al., 1993). Xid is a relatively mild immune deficiency with loss of some B-cell subpopulations and humoral responses (Scher, 1982). Allele-specific variation in disease severity seemed a plausible explanation until mice with null mutations of *Btk* were created and shown to have a mild xid-like phenotype (Hendriks et al., 1996; Khan et al., 1995), rather than the panhypogammaglobulinemia and profound loss of B cells seen in XLA. The difference in the severity of the xid defect compared to that seen in patients with XLA, and other studies described below strongly support the concept that the effects of the B-cell immune deficit due to loss of Btk function is strongly influenced by genetic context.

The xid mutation was first defined in the early 1970s as abnormal responses to polysaccharide antigens in the highly inbred CBA/N strain of mice, a defect that segregated in a sex-linked manner (Amsbaugh et al., 1972). This strain of mice and congenic derivatives of the xid mutation on other backgrounds show normal fecundity and have minimal if any elevation of cancer risk. They also do not have to be housed in specialized animal facilities.

Detailed analysis of the immune defects in xid show cell-autonomous defects only for the B-cell lineage. These include the loss of specific cell subpopulations such as $CD5^+$ or B1 cells that normally accumulate in the peritoneum. The remaining B cells that emigrate from the marrow to peripheral sites show an immature phenotype exemplified by a high IgM-to-IgD ratio, low major histocompatibility complex (MHC) class II antigen expression, and inability to secrete IgM or class switch for certain isotypes such as IgG3. In xid mice, responses to type II T-cell-independent antigens, exemplified by the trinitrophenyl-derivatived Ficoll, are absent, and B cells cannot form multicellular colonies in response to certain mitogens (Bona et al., 1980, Scher, 1982).

Responses to T-cell-dependent antigens are normal. If T-cell help is removed through neonatal thymectomy or because of the simultaneous presence of the nu/nu gene, B-cell development in the bone marrow is blunted, and the immune deficit is severe (Karagogeos et al., 1986; Wortis et al., 1982). The presence of the xid allele can relieve the systemic lupus-like autoimmune disease complex seen in mouse strains such as NZB/NZW (Taurog et al., 1979), reduce the susceptibility to *S. aureus*-induced arthritis (Zhao et al., 1995) and *M. pulmonis* pneumonia (Sandstedt et al., 1997), and ameliorate diabetes in the NOD mouse (Kendall et al., 2009). These effects can be at least partially explained by the altered production of pathogenic antibodies associated with the xid allele, but an altered balance of cytokines produced by B cells may also play a role. More subtle and complex genetic interactions are seen when the severity of the xid allele is evaluated in the context of specific backcrosses or different congenic strains that do not harbor any obvious immune or developmental defect.

Development of wild-type levels of Btk by transgenesis experiments can reconstitute normal B-cell function and development (Drabek et al., 1997). Several studies have combined the xid mutation with loss of function in a second gene to create more severe B-cell phenotypes and help define pathways that are likely rate-limiting for Btk function. A striking result is the severe block in B-cell development at the pre-B-cell stage observed when a loss of Btk function is combined with a loss of Tec function (Ellmeier et al., 2000). This can be most simply interpreted as Btk and Tec serving essential but redundant roles in the pathways connected to the pre-BCR. The phenotype of this mouse more closely resembles that seen in patients with XLA but is still not quite as severe.

Several strains of knockout mice result in a xid-like phenotype. Mutation affecting PLCγ2, the p85 form of PI3K, PKCβ, or the adaptor protein BLNK all show an xid-like phenotype with selective loss of B cells and antigen responses, defective activation from the Ig receptor, and loss of Ca^{2+} flux in residual peripheral B cells (reviewed in Fruman et al., 2000). The proteins encoded by these gene all work in a complex or signalosome that mediates signals from cell-membrane receptors, including but not limited to the BCR.

The concept that there is an optimal dose of Btk is supported by the phenotype of a transgenic strain of mice expressing a mutant (E41K) form of the enzyme (Dingjan et al., 1998; Maas et al., 1999). This mutation was first isolated by random mutagenesis and selection for an allele of Btk that could transform fibroblast cells to grow suspended in agar. The

mutation increases the ability of the enzyme to associate with membranes and the fraction of enzyme chronically modified by phosphotyrosine, indicating that it is an activated allele (Li et al., 1995). The transgenic E41K strain did not overproduce B cells or have hyperactivated B cells, but rather produced a more severe block in B-cell development at the pre-B-cell stage similar to that in the human XLA phenotype. However, this phenotype was seen only in transgenic animals carrying several copies of the mutated gene fragment, suggesting that this phenomenon would be highly unlikely to occur in human beings. This finding implicates that too much Btk activity creates negative signals, which are even more potent as inducers of phenotypic alterations as compared to loss-of-function mutations. *Drosophila melanogaster* also develop an overt phenotype upon transposon-mediated Btk loss-of-function mutations. These can be complemented by heat-shock-induced expression of human BTK (Hamada et al., 2005). Even if flies represent an interesting model for Btk-mediated signaling, the downstream pathway of Btk has been evolutionarily rewired (Nawaz et al., 2012).

ANIMAL MODELS OF AUTOSOMAL RECESSIVE AGAMMAGLOBULINEMIA

In the early 1990s Kitamura et al. (1991) produced a strain of mice, called μMT mice, that had a premature stop codon in the membrane exons of μ heavy chain. These mice, which were among the first knockout mice to be created, were able to make the secretory form of μ heavy chain but not the membrane form. By deleting all of the JH segments a second model of μ heavy chain deficiency was generated (Chen et al., 1993). In both strains, the earliest precursors of B cells could be found in the bone marrow, but there was a complete block in differentiation at the pre-B-cell stage, indicating that an intact membrane form of μ heavy chain is critical for early B-cell development. Chen et al. noted that the block in differentiation was identical to that seen in RAG1- or RAG2-deficient mice. The homozygous mutant mice had no measurable serum IgM or IgG and there were no B cells in the spleen, lymph nodes, or peritoneum. Heterozygous mice were normal. This phenotype is identical to that seen in patients with defects in μ heavy chain.

B-cell precursors from the JH deletion mice showed abundant production of VH sterile transcripts but no VH-to-DH rearrangement, supporting the hypothesis that DH-to-JH rearrangement must occur prior to VH-to-DH rearrangement. However, a low level of kappa chain V-to-J rearrangement was seen in both the μMT mice and the JH deletion mice, indicating that production of an intact membrane form of μ heavy chain is not required for light chain rearrangement.

Studies in the μMT mice showed that allelic exclusion, the exclusive production of a heavy chain protein from one of the two chromosomes, required the expression of an intact membrane form of μ heavy chain (Kitamura and Rajewsky, 1992). Mice that were heterozygous for the mutant μMT locus and a wild-type locus had B-lineage cells expressing secretory μ heavy chain from both alleles.

Later studies showed that the genetic background could influence the phenotype of the μMT mice. When the μMT mutation was placed on a Fas or Fas ligand-deficient background, normal or nearly normal concentrations of all IgG subclasses were produced (Melamed et al., 2000). These mice made several autoantibodies but they did not produce antigen-specific antibody in response to exogenous antigens. The original μMT mice were on a mixed 129/B6 background. When the μMT mutation was placed on the CBA/J background, there were no changes in the phenotype. However, when the mutation was placed on the BALB/C background, the serum IgA, IgG1, IgG2a, and IgG2b levels were within the normal range and the IgD and IgE levels were elevated compared to wild-type mice (Hasan et al., 2002). The serum IgM was undetectable and the IgG3 was significantly decreased. Backcross experiments indicated that the ability of BALB/C μMT mice to make IgG was controlled by a single autosomal dominant gene. Macpherson et al. (2001) reported that IgA production could be detected in the intestinal tract of μMT mice on the C57BL/6 background, but this finding was not seen by others (Hasan et al., 2002; Melamed et al., 2000). Careful search for IgA in the serum and intestines of patients with defects in μ heavy chain did not reveal any immunoglobulin production (Pan et al., 2002).

Many studies have used μ heavy chain-deficient mice to examine the role of B cells and/or antibody in protection from infection. The majority of these studies suggest that the lack of B cells exacerbates infection with parasites, like cryptosporidium or toxoplasma (Chen et al., 2003a, 2003b); viruses like West Nile virus, influenza, or adenovirus (Diamond et al., 2003; Graham and Braciale, 1997; Moore et al., 2004); and fungus (Montagnoli et al., 2003). None of these organisms causes significantly greater problems in B-cell-deficient patients compared to healthy controls. The μMT mice have also been used as a model for mycoplasma infections, which are more common and severe in patients with agammaglobulinemia and absent B cells (Berglöf et al., 1997; Roifman et al., 1986).

Several different strains of mice that are deficient in Igα or Igβ have been produced. Mice that are null for either or both of these genes have a block in B-cell differentiation at the same stage of development as mice with defects in μ heavy chain or RAG (Gong and Nussenzweig, 1996; Pelanda et al., 2002). By contrast, mice that express truncated forms of either Igα or Igβ, forms that lack the cytoplasmic ITAM motif, are able to generate immature B cells but have defects in later stages of maturation (Reichlin et al., 2001; Torres et al., 1996). However, in knockin mice with mutations in the ITAM motifs of both Igα and Igβ, the block in B-cell development is equivalent to that seen in μ heavy chain-deficient mice (Kraus et al., 2001). These findings indicate that Igα and Igβ are essential and nonredundant for the expression of the pre-BCR, and at least one ITAM motif is needed to support the pro-B-cell to pre-B-cell transition, but the signaling motif of either Igα or Igβ is sufficient for progression from pre-B cells to immature stages of B-cell development. Like the murine models, patients with null mutations in either Igα and Igβ have a complete block in B-cell development that is identical to that seen in patients with defects in μ heavy chain. There are no murine models for

alterations in the extracellular domains of Igα or Igβ, as was seen in one of the patients with an amino acid substitution in Igβ (Dobbs et al., 2007).

Mice with defects in λ5 have a milder immunodeficiency than patients with mutations in the same gene. At 6 weeks of age the mutant mice have approximately 5 percent of the normal number of B cells in the peripheral blood and 15 percent of the normal number of B cells in the spleen (Kitamura et al., 1992). However, with age, the number of B cells improves. At 4 months of age, the λ5-deficient mice have 20 percent of the normal number of B cells and normal amounts of serum IgM. These mice are able to make antigen-specific antibody in response to both T-cell-dependent and T-cell-independent antigens, although the titer of antibody to T-cell-dependent antigens is decreased. Mice that lack VpreB as well as λ5 have a phenotype that is very similar to that seen in mice that are deficient in λ5 alone (Shimizu et al., 1992). This suggests that neither VpreB nor λ5 has a function in the absence of its partner. Patients with defects in λ5 have a severe, profound defect in B-cell development that does not improve with age (Minegishi et al., 1998, and personal observation).

Several different strains of mice with mutations in BLNK (also called SLP-65 or BASH) have been produced (Hayashi et al., 2000; Jumaa et al., 1999; Pappu et al., 1999; Xu et al., 2000). All of these mice have a leaky block in B-cell differentiation at the pre-B-cell stage of development, but there is some variability in the severity of the defect in the different strains of mice. These differences may be due to the particular construct used to create the knockout, the genetic background of the mice, or the environmental conditions of the mice. Like the λ5-deficient mice, the BLNK-deficient mice demonstrated improved B-cell numbers with age (Pappu et al., 1999; Xu et al., 2000). Serum IgM and IgG3 levels are decreased or absent and the concentrations of IgG1, IgG2a, and IgG2b are less severely affected. Although the number of patients with mutations in BLNK is small, all of these patients have a defect that is more severe than the most severe BLNK-deficient murine stain, the strain produced by Pappu et al. (1999). Further, there has been no improvement in B-cell development with age in the BLNK-deficient patients.

It has been suggested that BLNKmay function as a tumor suppressor. There is a markedly increased incidence of pre-B-cell leukemia in the mildest strain of BLNK-deficient mice, the strain produced by Jumaa et al. (1999). If the BLNK mutation is placed on a BTK-deficient background, the incidence of leukemia increases further (Kersseboom et al., 2006). The Jumaa group reported that abnormal splicing of the BLNK message was seen in 16 of 34 patients with acute lymphocytic leukemia (ALL) (Jumaa et al., 2003). However, in a much larger series of patients with ALL, only 9 of 284 patients had decreased expression of BLNK as evaluated by the Affymetrix GeneChip (Imai et al., 2004). It may be that the increased incidence of leukemia seen in the BLNK-deficient mice is due to hyperproliferation of early B-cell precursors. It has been noted that many of the murine strains with blocks at the pre-B-cell stage of differentiation have increased numbers of pro-B cells (Jumaa et al., 1999; Pappu et al., 1999). This might be attributed to a positive feedback signal responding to the low B-cell numbers in the periphery. Patients with early defects in B-cell development do not show an increased number of pro-B cells, perhaps because the niche for pro-B cells is more limiting in the human compared to the mouse.

CONCLUDING REMARKS

Although *BTK* was identified as the gene responsible for XLA two decades ago, much still needs to be learned about the function of this molecule. For example, BTK is clearly required for signaling through the BCR; however, the other signaling pathways that influence BTK function are less clear. We know that there are modifying genetic factors that influence the severity of XLA. What are these factors? It is likely that some of them are intrinsic to the B cell. Others may be extrinsic to the B-cell lineage and influence B-cell homeostasis or survival; some may provide alternative routes for protection from infection. Understanding the modifying genetic factors that make some patients more susceptible to enteroviral infection or inflammatory bowel disease may help us treat those patients.

As noted above (see the section "Activation of BTK by Receptors in Non-B Cell S"), absence of BTK can result in monocyte and platelet abnormalities that can be demonstrated in the laboratory; however, it is not clear if there are clinically significant effects in these cell lineages. Comparison of clinical complications seen in patients with *BTK* mutations with patients who have mutations in one of the B-cell-specific genes, for example μ heavy chain, may begin to answer this question.

Murine models of disease have proved to be very useful in understanding the framework of B-cell development. However, findings in mice cannot be directly extrapolated to humans. Mutations affecting BTK, λ5, and BLNK result in a more severe phenotype in humans compared to mice. What is the basis of this difference? Could it be due to differences in positive or negative selection at early stages of B-cell development? Or perhaps it is due to differences in the bone marrow niche that supports the pro-B-cell to pre-B-cell transition.

The prognosis for patients with XLA or ARA has improved each decade over the past 50 years due to earlier age at diagnosis, improved preparations of gammaglobulin, and more effective antibiotics. Yet further improvements are needed. These diseases are still associated with a significant incidence of complications and they are very expensive to treat. Patients and their families are eager for new treatment modalities that "fix" their immunodeficiency.

It is also interesting to note that inhibitors of BTK have become increasingly useful in the treatment of leukemia, lymphoma, and autoimmunity. On a global scale it does not seem unlikely that patients receiving BTK inhibitors eventually will outnumber those with inherited defects by orders of magnitude.

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26.

CD40 AND CD40 LIGAND DEFICIENCIES

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INTRODUCTION

The primary B-cell repertoire is generated in the bone marrow by means of antigen-independent stochastic rearrangement and assembly of Variable (V), Diversity (D), and Joining (J) gene elements of immunoglobulin heavy and light chain genes. In the periphery, two key events mark maturation of the antibody response upon antigen encounter: class-switch recombination (CSR) and somatic hypermutation (SHM). During CSR, the mu heavy chain is replaced by a downstream constant heavy chain gene element. This process results from deletional recombination between characteristic repetitive sequences (switch regions) located 5′ of the Cμ gene and of each C_H gene, except Cδ. Replacement of the μ heavy chain with other constant heavy chain elements has a significant impact on the biological properties of the immunoglobulin that is produced. SHM, on the other hand, consists of the introduction of nucleotide substitutions in the immunoglobulin V regions and may thus cause changes in the amino acid sequence and in the affinity of the immunoglobulin for the antigen. As a result of this process, B-cells expressing surface immunoglobulins with higher affinity for the antigen are positively selected during the germinal center reaction.

A variety of defects of CSR are known in humans and are characterized by recurrent infections and very low levels of serum IgG, IgA, and IgE, with normal or elevated IgM (Notarangelo et al., 2006). Both primary and acquired forms of the disease have been reported. Among primary CSR defects, X-linked, autosomal recessive, and (less frequently) autosomal dominant forms have been reported, reflecting genetic heterogeneity. Defects of CSR are often associated with abnormalities of SHM and of generation of memory B-cells. Based on the underlying cellular pathophysiology, CSR defects can be divided into B-cell–intrinsic and B-cell–extrinsic disorders. The latter group includes an X-linked variant of the disease, due to mutations of the *CD40LG* gene, that encodes for CD40 ligand (also known as CD154), a molecule predominantly expressed by activated $CD4^+$ T lymphocytes. CD40LG interacts with CD40, which is constitutively expressed by B-cells. CD40LG/CD40 interaction elicits B-lymphocyte intracellular signaling, with induction of the NF-κB signaling pathway and expression of activation-induced cytidine deaminase (AICD) and uracil N-glycosylase (UNG), two B-cell–specific enzymes that play a key role in CSR and SHM (Notarangelo et al., 2006). In humans, mutations of *CD40LG*, *CD40*, *IKBKG* (encoding for IKK-γ/NEMO, a regulator of the NF-κB signaling pathway), and *AICD* and of the *UNG* genes are all associated with defective CSR. In this chapter, we will focus on CD40LG and CD40 defects. Deficiencies of AICD, UNG, and IKK-γ/NEMO will be discussed in Chapters 27 and 36, respectively.

BIOLOGY OF CD40 AND CD40 LIGAND

EXPRESSION AND BIOLOGICAL ROLE OF CD40

CD40, a 50kDa glycoprotein, is a member of the tumor necrosis factor receptor (TNFR) family of surface molecules. The gene, *CD40* (*109535), encodes a Cys-rich type I transmembrane protein of 277 amino acids (a.a.) (Stamenkovic et al., 1989). CD40 is constitutively expressed on all B-cells but also on other cell types, including dendritic cells (DCs), monocytes, and macrophages (Brouty-Boye et al., 2000; van Kooten and Banchereau, 2000). Expression of CD40 has been demonstrated also on $CD34^+$ hematopoietic precursor cells (Pyrovolaki et al/, 2009), T lymphocytes (Munroe et al., 2007; Vaitaitis et al., 2008), and platelets (Inwald et al.,

2003). Finally, CD40 is also expressed by nonhematopoietic cell types such as thymic epithelial cells, some other epithelial and endothelial cells, and neurons (van Kooten and Banchereau, 2000).

CD40 plays an important role in B-cell survival, growth, and differentiation. CD40 ligation on the surface of B-cells in the presence of interleukin-4 (IL-4) initiates proliferation and growth and induces homotypic cell adhesion and upregulation of the expression of CD23, CD54, CD80, CD86, CD95 (Fas), and lymphotoxin-α. CD40-mediated upregulation of Fas expression on B-cells makes them susceptible to killing by Fas ligand$^+$ (FasL$^+$)-activated T-cells (Banchereau et al., 1994; van Kooten and Banchereau, 2000). This may play a role in the CD40-mediated apoptosis of tumor B-cells (Georgopoulos et al., 2006) and in peripheral purging of self-reactive B lymphocytes, as indicated by the increased frequency of self-reactive B-cells in patients with CD40LG deficiency (Hervé et al., 2007). However, CD40 also delivers an antiapoptotic signal to B-cells by inducing antiapoptotic genes that include Bcl-xL and A20 (Ishida et al., 1995; Sarma et al., 1995). It has been shown that C4 binding protein (C4BP), a regulator component of the classical complement pathway, may also bind to CD40 on human B-cells at a site that differs from that used by CD40LG. Engagement of CD40 by C4BP triggers signaling pathways similar to those triggered by CD40LG, as demonstrated by the fact that this interaction induces proliferation, upregulation of CD54 and CD86 expression, and IL-4–dependent IgE isotype switching in normal B-cells (Brodeur et al., 2003). IL-4 and IL-13, the switch factors for IgE, induce the transcription of a 1.8 kb ε germline mRNA that initiates 5′ of the Sε region. This transcript is sterile, as it is not translated into a functional protein. Induction of a mature 2.0 kb ε mRNA and of IgE protein synthesis requires a second signal, provided by T-cells, via CD40LG/CD40 interactions (Oettgen, 2000). *Cd40*$^{-/-}$ mice fail to undergo T-cell–dependent isotype switching and fail to develop germinal centers following immunization with T-cell–dependent antigens (Castigli et al., 1994; Kawabe et al., 1994). Similar results were obtained in mice with disrupted *CD40LG* genes (Borrow et al., 1996; Xu et al., 1994). Cross-linking of CD40 in the presence of IL-4 induces the expression of AICD. AID has a critical role in CSR, as demonstrated by the fact that B-cells from AID$^{-/-}$ mice and from patients with AID deficiency are unable to undergo isotype switching (Muramatsu et al., 2000; Revy et al., 2000). Several molecules that play a role in DNA repair have been found to be important or essential for isotype switching; these include Ku70, Ku80, DNA-PK, Msh2, Pms2, and others (Stavnezer et al., 2008).

CD40/CD40LG interaction is also important for the maturation of myeloid DCs (marked by upregulation of CD80, CD83, CD86, and CD203) and for IL-12 production (Cella et al., 1996; Fontana et al., 2003). CD40LG-expressing CD8$^+$ T-cells activate CD8α$^+$ DC for IL-12 p70 production during antigen-specific T-cell responses and favor cross-presentation and cross-priming of cytotoxic T-cells (Wong et al., 2008). In addition, engagement of CD40 on the surface of plasmocytoid DCs drives a potent Th1 polarization and promotes interferon (IFN)-α secretion in response to viral infections (Cella et al., 2000). In keeping with these observations, both maturation and activation of myeloid and plasmocytoid DCs are severely impaired in patients with CD40 deficiency (Fontana et al., 2003).

CD40 plays also a critical role in the induction of inflammatory responses and in thrombosis. Both CD40 and CD40LG are expressed on the surface of platelets. CD40LG/CD40-mediated platelet–platelet and platelet–lymphocyte interactions favor recruitment of leukocytes to sites of thrombosis or inflammation (Li, 2008). It has recently been shown that CD40 may also be expressed by T-cells, in particular activated CD4$^+$ T lymphocytes, and that it can act as a co-stimulatory molecule and synergize with CD28 during TCR-mediated activation (Munroe et al., 2007).

CD40 plays an important role also in establishing the thymic medullary microenvironment and self-tolerance. In postnatal life, cooperation between CD40- and RANK-mediated signaling was found to be required to support differentiation and maintenance of thymic medulla in mice through a TNF-associated factor 6 (TRAF-6)-, NF-κB inducing kinase (NIK)-, and IκB kinase β (IKKβ)-dependent manner (Akiyama et al., 2008). In this process, CD40 on the surface of medullary thymic epithelial cells (mTECs) is engaged by CD40LG expressed by positively selected CD4$^+$ thymocytes that bear self-reactive TCR-recognizing self-antigens expressed by mTECs in the context of major histocompatibility complex (MHC) class II molecules (Irla et al., 2008). Furthermore, negative selection of endogenously expressed antigens and superantigens is blocked by the administration of antibodies to CD40 ligand (Foy et al., 1995). Altogether, these findings establish a critical role for CD40 in central tolerance.

REGULATION OF CD40 EXPRESSION AND SIGNIFICANCE OF CD40 VARIANTS

In humans, transcription of the *CD40* gene results in production of a 277-a.a. protein, which contains of a 22-a.a. signal sequence, a 171-a.a. extracellular domain, a 22-a.a. transmembrane domain, and a 62-a.a. cytoplasmic domain. Murine CD40 is a 289-a.a. protein and includes a C-terminal 11-a.a. sequence that is not present in human CD40. Regulation of CD40 expression and function is driven by posttranscriptional and posttranslational mechanisms.

CD40 expression is regulated at the transcriptional level by the AT-hook transcription factor AKNA. This factor is known to bind A/T-rich regulatory elements of the promoter DNA and to change their architecture, increasing promoter accessibility. This transcription factor coordinately regulates CD40LG expression as well and thus is important to promote homotypic cell interactions (Siddiqa et al., 2001).

Regulation of CD40 expression is achieved also at the posttranscriptional level through alternative splicing. Modulation of CD40 mRNA isoform expression appears to play an important role in the regulation of CD40LG-mediated signaling. Five different CD40 splicing variants are differentially expressed in activated murine macrophages and DCs (Tone et al., 2001). The type I isoform corresponds to the full-length, functional mRNA and is also the most abundant

splicing variant. A C-terminal truncated *CD40* mRNA isoform (type II) represents the major alternative splicing variant and encodes for a protein unable to deliver intracellular signaling. Type III and IV mRNA products also encode for nonfunctional protein and may act through a dominant negative mechanism by forming nonfunctional trimers with type I CD40 protein. Differential expression of *CD40* mRNA splicing variants during brain development has been implied to play an important role in modulating neuronal differentiation, and CD40-deficient mice have aberrant neuron morphology and gross brain abnormalities (Hou et al., 2008).

In humans, various alternative CD40 splice variants have been also described. In particular, three splice variants have been identified that differ from full-length *CD40* mRNA because they lack exon 5, exon 6, or both exons 5 and 6. Using computational biology tools, the first two of these variants were predicted to encode for soluble decoy receptors because of the lack of the transmembrane domain. Indeed, soluble forms of CD40 have been detected in the serum of uremic patients, but only the isoform lacking the a.a. encoded by exon 6 has the ability to bind CD40LG and may thus serve as a true decoy receptor (Eshel et al., 2008).

Several variants have been identified in the *CD40* gene, and some of these have been associated with an increased risk to develop certain diseases. Association of *CD40* single nucleotide polymorphisms (SNPs) with autoantibody-positive rheumatoid arthritis (possibly involving increased NF-κB-mediated signaling) has been reported (Raychaudhuri et al., 2008). This association suggests that CD40 signaling could mediate rheumatoid pathogenesis through NF-κB activation. Contradictory data are available on the association between CD40 known variants (particularly one in the Kozak fragment) and increased susceptibility to Graves disease, with some evidence that increased thyroidal expression of mutated CD40 may contribute to this disease specificity (Jacobson et al., 2007; Kurylowicz et al., 2005). The same intronic variant has been associated with an increased risk of follicular lymphoma associated with lower expression of CD40 molecule (Skibola et al., 2008).

Some of these SNPs have been characterized from the functional point of view. The P227A CD40 variant resides in the cytoplasmic domain, has been found only in heterozygosity, and has a frequency of 29 percent in South American–descent subjects and less than 1 percent in all the other ethnic groups. This variant has been found to result in increased IgM production, augmented secretion of IL-6 and TNF-alpha, and phosphorylation of the JNK (MAPK8) target, c-Jun, even if binding affinity for TRAF molecules is similar to that reported for wild-type CD40 (Peters et al., 2008).

A single nucleotide change, resulting in an a.a. substitution (p.H78Q) in the second cysteine-rich extramembrane region of CD40, has been described in a multiple myeloma cell line (U266) and in freshly isolated tumor cells. This mutant protein has unique functional properties, including decreased binding affinity for CD40LG and constitutive association with TRAF6 in the absence of CD40LG stimulation. The overall survival rate of patients with multiple myeloma carrying this CD40 mutation was significantly poorer than those expressing wild-type CD40, suggesting that the mutated protein has a role in tumor invasion and metastasis and that CD40/CD40LG interaction may modulate tumor pathogenesis and/or progression (Qi et al., 2009).

CD40-MEDIATED SIGNALING

A schematic representation of CD40-mediated signaling pathways is shown in Figure 26.1. CD40 lacks intrinsic catalytic activity, but its cytoplasmic domain contains binding motifs for several signal-transducing molecules, thus permitting CD40-mediated regulation of humoral and cellular immunity processes (Elgueta et al., 2009). In particular, CD40 binds Jak3 in its proline-rich Box 1 membrane proximal region (a.a. 222–229) and has one additional binding site for TRAF-6 and one for TRAF-2/3/5 proteins (Hanissian and Geha, 1997). Recently, another TRAF-2 binding site has been identified, which is also involved in mediating B-cell activation, proliferation, and differentiation (Lu et al., 2007). CD40 ligation by membrane-bound trimeric CD40LG causes a higher degree of oligomerization and, most importantly, a conformational change that results in translocation of CD40 and associated TRAF proteins to lipid rafts (Xia et al., 2007). When TRAF proteins bind to the cytoplasmic tail of CD40, various signaling cascades are activated, including JAK3-STAT5, NF-κB, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinase pathways (Jabara et al., 2009; Jalukar et al., 2000; Revy et al., 1999; Zarnegar et al., 2004). In B lymphocytes, CD40-induced activation of TRAF-6 is required for IgM production and isotype switching, whereas CD40 interaction with TRAF-2/3/5 promotes CD80/CD86 upregulation and protection from B-cell-antigen-receptor–mediated growth arrest (Ahonen, 2002).

TRAF2 and TRAF6 play an important physiological role in CD40 signaling also in nonhematopoietic cells, and their role is mutually linked, as TRAF6 regulates CD40 signal transduction not only through its direct binding to CD40 but also indirectly via its association with TRAF2 (Davies et al., 2005). A truncated derivative of TRAF2 lacking an amino-terminal RING finger domain is a dominant-negative inhibitor of NF-κB activation mediated by CD40 (Rothe et al., 1995). An 11-a.a motif in the intracellular domain of CD40 that spans the core Px-QxT TRAF2,3 binding sequence was found to be sufficient for the activation of Jun, p38, and NF-κB. A CD40 mutant that binds TRAF2 but not TRAF3 (or TRAF5) was shown to activate NF-κB. This finding, together with the inability of TRAF1 to activate NF-κB, suggests that TRAF2 is the most important element in CD40-mediated activation of NF-κB. TRAF2 has also been found to bind to the kinase NIK, which can also phosphorylate and activate the IKK complex. TRAF2 also binds to RIP, a protein that is central to NF-κB activation by TNFR1 and to the protection of cells from TNF-mediated death. RIP binds to MEKK3, which then phosphorylates and activates IKK (Wang et al., 2001). It has been shown that TRAF6 functions as an E3 ubiquitin ligase to catalyze, together with Ubc13/Uev1A, the synthesis of polyubiquitin chains linked through lysine-63 (K63) of ubiquitin and the activation of a TAK1–TAB1/2 complex,

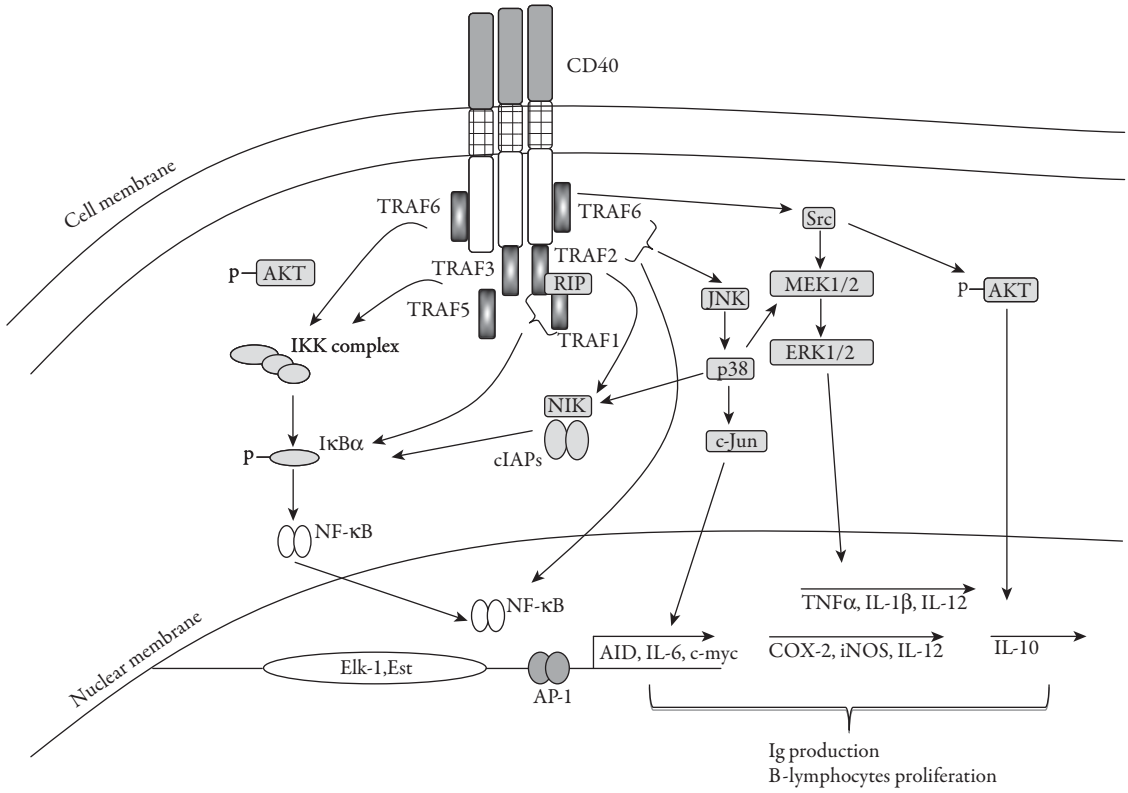

Figure 26.1 Hypothetical model of CD40 signaling. CD40 ligation causes enhanced association of CD40 with tumor necrosis factor receptor-associated factor (TRAF) proteins, resulting in activation of various signaling pathways. In B lymphocytes CD40-induced activation results in transcription of critical genes involved in B-cell proliferation and terminal B-cell differentiation. TRAF2 and TRAF6 interact and activate both the NF-κB and the JNK/p38 pathways. NF-κB activation is regulated via the binding with the RIP protein. Various signaling pathways induce phosphorylation of the IKK complex, leading to nuclear translocation of NF-κB and transcription of target genes.

which phosphorylates and activates IKK (Deng et al., 2000; Shuto et al., 2001; Yang et al., 2000).

Data from knockout mice and from patients with IKKγ/NEMO mutations (Döffinger et al., 2001; Jain et al., 2001; Zonana et al., 2000) strongly suggest that NF-κB plays an important role in CD40-mediated isotype switching. Traf2$^{-/-}$ mice die perinatally, but Traf2$^{-/-}$Tnfr1$^{-/-}$ and Traf2$^{-/-}$ Tnf$^{-/-}$ double-mutant mice are viable (Yeh et al., 1997). Isotype switching to IgG is impaired in Traf2$^{-/-}$ Tnfr1$^{-/-}$ mice. This finding further supports the idea that a pathway consisting of TRAF2-mediated activation of NF-κB in CD40-activated B-cells is critical for isotype switching. Two different NF-κB activation pathways have been described: type 1 (p50-dependent) and type 2 (p52-dependent). In resting B-cells, the alternative NK-κB signaling pathway is inhibited by TRAF2, TRAF3, cIAP1, and cIAP2 by targeting and ubiquitin-dependent degradation of NIK (NF-κB-inducing kinase), thus preventing cleavage of p100 precursors. CD40 and B-cell activating factor receptor (BAFF-R) engagement results in TRAF3 degradation, which blocks association of NIK with the cIAP1–cIAP2–TRAF2 ubiquitin ligase complex and p100 processing and hence enables activation of the type 2 NF-κB signaling pathway (Vallabhapurapu et al., 2008). CD40 and lipopolysaccharide (LPS) can both mediate activation of the type 1 NF-κB signaling pathway. The observation that CD40LG/CD40 interaction promotes homotypic cell adhesion, whereas neither LPS nor BAFF is proficient in this process, indicates that simultaneous activation of type 1 and type 2 NF-κB signaling pathways is required to induce homotypic B-cell adhesion (Zarnegar et al., 2004). Finally, the redox factor APE/Ref-1 has been reported to act as a key signaling intermediate in response to CD40-mediated B-cell activation. Upon CD40 cross-linking, TRAF2 adapter is involved in APE/Ref-1 translocation from the cytoplasm to the nucleus, where it modulates the DNA-binding activity of the Pax5 and EBF transcription factors. APE/Ref-1 appears to be required for CD40-mediated Pax5 activation, as the repression of APE/Ref-1 protein production is able to block CD40-induced Pax5 binding activity (Merluzzi et al., 2008).

CD40 LIGAND

The murine CD40LG has been identified and the gene cloned as an EL4 thymoma cell surface molecule that binds to soluble CD40 (Armitage et al., 1992). Subsequently, the human *CD40LG* gene (*300386), encoding for CD40LG (CD154), was cloned from activated T-cells (Graf et al., 1992; Hollenbaugh et al., 1992; Spriggs et al., 1992). Independently, Lederman et al. identified a subclone of Jurkat T-cells that is able to provide contact-dependent helper function to B-cells (Lederman et al., 1992); the molecule responsible for this helper activity was cloned and confirmed to be CD40LG. The human

cDNA has an open reading frame of 783 base pairs (bp) that codes for a type II membrane protein 261 a.a. long. The extracellular domain is 215 a.a. long, the transmembrane domain is 24 a.a. in length, and the intracellular domain is 22 a.a. long. The presence of a proteolytic cleavage site (His-Arg-Arg-Leu) immediately proximal to the transmembrane domain allows production of soluble forms of CD40LG. Platelets represent the major source of soluble CD40LG in the blood, and recent data indicate that the matrix metalloproteinase-2 (MMP-2) might be the protease primarily responsible for CD40LG cleavage from platelet surface (Reinboldt et al., 2009). Molecular modeling of CD40LG based on the crystal structure of TNF indicates that a.a. residues 189 to 209 are critical for the binding of CD40LG to CD40 (Peitsch and Jongeneel, 1993).

Although de novo synthesis of CD40LG occurs following antigen recognition by T lymphocytes, surface mobilization of preformed, intracellular CD40LG has been also observed in both effector and memory $CD4^+$ T-cells following antigen recognition. Intracellular CD40LG is stored in secretory lysosomes and co-localizes more strongly with Fas ligand than with CTLA-4, two other molecules that are delivered to the cell surface following antigen recognition (Koguchi et al., 2007).

Detailed organization of the murine (Tsitsikov et al., 1994) and human (Shimadzu et al., 1995; Villa et al., 1994) *CD40LG* genes has been reported. The gene consists of five exons. Exon 1 encodes for the 5′ untranslated region and the first 52 a.a., including 22 a.a. of the intracytoplasmic tail, 24 a.a. of the transmembrane domain, and 6 a.a. of the extracellular domain. Exons 2, 3, and 4 encode a.a. 53 to 96, a.a. 97 to 114, and a.a. 115 to 135, respectively. Exon 5 encodes the rest of the protein (a.a. 136–260) and the 3′ untranslated region. There is no homology in the position of the exons between CD40LG and any of the known genes from the TNF family, but in all these genes the sequence encoding the receptor binding domain is located in the last exon.

Several studies have illustrated the importance of CD40 and its ligand, CD40LG, in humoral immunity. Recombinant CD40LG mimics the action of CD40 monoclonal antibody (MAb) and is able to stimulate B-cell proliferation in the presence of phorbol myristate acetate (PMA) and to induce immunoglobulin synthesis (Lane et al., 1993; Spriggs et al., 1992). Immunoglobulin secretion by B-cells is highly modulated by cytokines. In the presence of recombinant CD40LG, IL-2 and IL-10 induce specifically the secretion of IgM, IgG1, and IgA (Durandy et al., 1993), whereas IL-4 is necessary for the secretion of IgG4 and IgE. Antibody to CD40LG can block T-cell–dependent B-cell activation (Noelle et al., 1992a, 1992b). Immunization of mice with KLH (a thymus-dependent antigen) or DNP-Ficoll (a thymus-independent antigen) induces the expression of CD40LG by T helper (Th) cells, and this induction coincides with the development of cytokine-producing cells. Through the use of immunocytochemical techniques, it was elegantly demonstrated that CD40LG cells and cytokine-producing cells are juxtaposed in the lymphoid organs (Van den Eertwegh et al., 1993). Short-term treatment with anti-CD40LG antibody suppressed the immune response against these antigens, but adoptive transfer of cells from anti-CD40LG–treated mice could fully reconstitute Th-cell function in irradiated recipient mice, showing that immune suppression does not involve clonal anergy or deletion (Foy et al., 1993). Thus it appears that CD40LG expression is essential for both thymus-dependent and thymus-independent antigens. However, in mice with targeted disruption of the *Cd40* or *Cd40lg* gene, only the antibody response against the T-dependent antigen is impaired. This finding suggests that stimulation via CD40/CD40LG may not be critical for antibody responses to T-independent antigens.

As reported above, CD40LG is also essential for the maturation of myeloid DCs and the activation of plasmacytoid DCs. In addition, it promotes macrophage effector function, as shown by the fact that $Cd40lg^{-/-}$ mice are less effective in stimulating allogenic macrophages to produce inflammatory cytokines and reactive nitrogen intermediates (Stout et al., 1996). CD40LG/CD40-dependent interaction between activated $CD4^+$ T-cells and DCs is also essential for efficient T-cell priming and licensing of cytotoxic $CD8^+$ T lymphocytes. On the other hand, recent data have shown that stimulation of TLR3 and TLR9 with agonists and certain viruses (such as influenza virus) can induce CD40LG expression of the surface of DCs and may promote efficient priming of cytotoxic $CD8^+$ T-cells, even in the absence of $CD4^+$ T lymphocytes (Johnson et al., 2009). Finally, $CD40LG^+$ T-cells that are generated under strong antigenic stimulation, in concert with certain microbial stimuli, synergistically increase DC IL-6 production and Th17 polarization. CD40-deficient DCs exhibit reduced cytokine release and fail to drive Th17 development in vitro (Iezzi et al., 2009).

REGULATION OF CD40 LIGAND EXPRESSION

CD40LG is a highly inducible gene and is mainly expressed by $CD4^+$ T-cells, although it can also be expressed by other cell types, including $CD8^+$ cells, monocytes, natural killer (NK) cells, and megakaryocytes (Cocks et al., 1993; Crist et al., 2008). In addition, masT-cells, basophils (Gauchat et al., 1993b), and eosinophils (Gauchat et al., 1995) can also express functional CD40LG. The T-cell expression of CD40LG is well regulated; resting T lymphocytes do not express CD40LG. In α/β T-cells, *CD40LG* mRNA becomes detectable as early as 1 hour after stimulation with PMA and ionomycin, peaks at 3 hours, and disappears by 24 hours. Surface CD40LG is detected as early as 3 hours after stimulation with PMA and ionomycin, peaks at 6 hours after stimulation, starts to decline by 8 hours, and is barely detectable by 16 hours. However, activation with anti-CD3 results in sustained expression of CD40LG for 24 hours after stimulation (Castle et al., 1993; Roy et al., 1993). Simultaneous engagement of the T-cell co-stimulatory molecule CD28 by mAb enhances CD40LG expression (Ding et al., 1995; Klaus et al., 1994). Highly purified peripheral blood $\gamma\delta$ T-cells also express CD40LG after stimulation (Horner et al., 1995). The kinetics of mRNA synthesis and surface expression closely resembles that of $\alpha\beta$ T-cells, but the levels are much lower. Prolonged *CD40LG* mRNA half-life has been observed following activation of antigen-primed T-cells as compared to naïve T-cells, indicating that posttranscriptional mechanisms play an important role in controlling CD40LG expression

during immune responses. In particular, it has been demonstrated that a CU-rich site within the 3′-untranslated region of CD40LG mRNA binds to a polypyrimidine tract-binding protein-containing complex (mComplex I) that is activation dependent (Vavassori et al., 2009).

CD40LG expression is developmentally regulated. Newborn T-cells were found to be deficient in CD40LG expression and in their ability to induce isotype switching in B-cells (Brugnoni et al., 1994; Durandy et al., 1995; Fuleihan et al., 1994; Nonoyama et al., 1995), and reduced CD40LG expression persists for the first 6 months of age (Gilmour et al., 2003).

Cyclosporin A (CsA) inhibits the surface and mRNA expression of CD40LG in human (Fuleihan et al., 1994) and murine (Roy et al., 1993) T-cells. CsA is a naturally occurring immunosuppressant that binds to its cellular receptor(s), cyclophilin, forming a complex that inhibits the activity of the phosphoprotein phosphatase calcineurin. Calcineurin dephosphorylates the cytoplasmic subunit of the transcription factor, NFAT, which translocates into the nucleus to form the functional NFAT complex and regulates the expression of the gene encoding IL-2 (Jain et al., 1993). The transcription of *CD40LG* mRNA and the surface expression of the protein are inhibited by pretreatment of T-cells with CsA in a dose-dependent manner. The ability of CsA analogs to inhibit CD40LG expression correlated with the affinity of the cyclophilin–drug complex to calcineurin and not with the affinity of the drug to cyclophilin. These results suggest that transcription factors activated by calcineurin, such as NFAT, regulate the transcription of the *CD40LG* gene, and this has been experimentally confirmed (see below).

Hydrocortisone upregulates *CD40LG* mRNA and protein expression in peripheral blood mononuclear cells (PBMCs) and induces IgE synthesis in IL-4–stimulated normal human B-cells. Disruption of CD40LG–CD40 interaction by soluble CD40-Ig fusion protein or anti-CD40LG mAb blocks the capacity of hydrocortisone to induce IgE synthesis in normal B-cells. Upregulation of *CD40LG* mRNA and induction of IgE synthesis by hydrocortisone were inhibited by the steroid hormone receptor antagonist RU-486 (Jabara et al., 2001). These results indicate that ligand-mediated activation of the glucocorticoid receptor (GR) upregulates CD40LG expression in human lymphocytes. It is possible that hydrocortisone acts by inducing GR binding to glucocorticoid-responsive elements present in the *CD40LG* promoter.

The organization of the murine and human *CD40LG* genes shows remarkable conservation even in the 5′ upstream regulatory sequences. The transcription originates from a G residue 68 bp upstream from the A of the initiation codon in both species (Schubert et al., 1995; Tsitsikov et al., 1994). Sequences up to 1.5 kb upstream from the murine gene lack the TATAA or CCAAT boxes but have an Sp1 sequence, six NFAT-like sequences, and one OAP-like site. Three of the NFAT-like consensus sequences (including the two most proximal ones) are conserved in the human *CD40LG* gene. The most proximal NFAT-binding motifs of the murine gene form two complexes as detected by electrophoretic mobility shift analysis (EMSA). Both complexes contain NFATc and NFATp and are sensitive to CsA (Tsytsykova et al., 1996). Similar findings have been reported for the human *CD40LG* promoter (Schubert et al., 1995).

A T-cell–specific *CD40LG* transcriptional enhancer is located upstream of the promoter. This enhancer binds NFAT1 and the Th2 transcription factor GATA-3 (Brunner et al., 2008). The 3′ untranslated region of the murine gene has two adjacent microsatellite repeats (a 50-bp-long CT and a 90-bp-long CA repeat) as well as two ATTTA elements that are putatively responsible for the stability of mRNA.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF CD40 LIGAND DEFICIENCY

CD40LG deficiency is characterized by an X-linked pattern of inheritance and clinical features that are suggestive of a combined immunodeficiency. Most patients with CD40LG deficiency present in infancy with recurrent upper and lower respiratory tract infections and have a unique predisposition to *Pneumocystis jiroveci* pneumonia, which may even mark the clinical onset of the disease (Levy et al., 1997; Marshall et al., 1964; Ochs and Wedgwood, 1989; Winkelstein et al., 2003). The frequency of *P. jiroveci* pneumonia in CD40LG deficiency varies from 31.7 to 48.1 percent in the European and U.S. series of patients (Winkelstein et al., 2003; L. Notarangelo et al., unpublished observation). Lung infections due to cytomegalovirus, respiratory syncytial virus, cryptococcus, and mycobacteria, including bacillus Calmette-Guérin, have been also reported and may lead to disseminated disease (Banatvala et al., 1994; Fremerey et al., 2009; Hostoffer et al., 1994; Iseki et al., 1994; Kyong et al., 1978; Levy et al., 1997; Tabone et al., 1994; Tu et al., 1991). An increased risk for paracoccidioidomycosis and other unsual infections has been recently reported (Cabral-Marques et al., 2012).

Diarrhea occurs in over 50 percent of CD40LG-deficient patients (Levy et al., 1997; Notarangelo et al., 1992; Winkelstein et al., 2003) and may become chronic and require total parenteral nutrition. Chronic watery diarrhea is often associated with *Cryptosporidium* infection (Stiehm et al., 1986), which may also contribute to sclerosing cholangitis, a severe and often fatal complication (Banatvala et al., 1994; DiPalma et al., 1986; Hayward et al., 1997; Levy et al., 1997; Winkelstein et al., 2003). The incidence of liver and biliary tract disease increases with age (Hayward et al., 1997).

Oral ulcers and proctitis are common manifestations (Banatvala et al., 1994; Benkerrou et al., 1990; Hong et al., 1962; Kyong et al., 1978; Macchi et al., 1995; Notarangelo et al., 1992; Rieger et al., 1980) and are usually associated with neutropenia, either chronic (Aruffo et al., 1993; Levy et al., 1997) or cyclic (Notarangelo et al., 1992; Shimadzu et al., 1995; Wang et al., 1994).

CD40LG-deficient patients have osteopenia, with lower bone mineral density and elevated levels of N-terminal telopeptides of type I collagen, a urinary marker indicative of osteoclast activity. Osteoclast differentiation of myeloid cells is induced by RANKL, and osteoclastic activity is modulated by IFN-γ. $CD4^+$ CD40LG-deficient human T-cells have normal

expression of RANKL and promote marked osteoclastogenesis of myeloid cells; however, this activity cannot be properly modulated because of impaired T-cell priming and reduced IFN-γ production (Lopez-Granados et al., 2007).

Patients with *CD40LG* mutations are also at increased risk for lymphomas (Filipovich et al., 1994) and liver/biliary tract and gastrointestinal tumors (including peripheral neuroectodermal tumors), which are rarely observed in other primary immunodeficiencies (Erdos et al., 2008; Facchetti et al., 1995; Hayward et al., 1997).

Autoimmune manifestations (arthritis, thrombocytopenia, hemolytic anemia, hypoparathyroidism, immune complex–mediated nephritis, and retinal pigment epithelium hypersensitivity) may occur in CD40LG deficiency, although they are less common than in autosomal forms of CSR defects (Benkerrou et al., 1990; Hollenbaugh et al., 1994, Pascual-Salcedo et al., 1983; Schuster et al., 2005). Anemia may also be secondary to chronic infections or to parvovirus B19-induced red blood cell aplasia. The latter may even be the only manifestation of disease in patients with a mild phenotype (Blaeser et al., 2005; Seyama et al., 1998a).

Severe neurological involvement has also been reported. Meningoencephalitis due to enterovirus infection may occur, despite regular administration of intravenous immunoglobulins (IVIG) (Cunningham et al., 1999; Halliday et al., 2003). Cerebral toxoplasmosis and progressive multifocal leukoencephalopathy, in some cases caused by reactivation of the human neurotropic JC virus, have been reported as the first sign of disease in adults with hypomorphic *CD40LG* mutations (Aschermann et al., 2007; Suzuki et al., 2006; Yong et al., 2008).

Lymph nodes of CD40LG-deficient patients lack germinal centers (Facchetti et al., 1995; Hong et al., 1962; Rosen et al., 1961; Stiehm and Fudenberg, 1966) (Color Plate 26.I). This is the consequence of ineffective CD40–CD40LG interaction in the extrafollicular areas, resulting in poor recruitment of germinal-center precursors. In addition, severe depletion and phenotypic abnormalities of follicular DCs have been reported that may contribute to poor antigen trapping and result in inefficient rescue of the few germinal-center B-cells from apoptosis (Facchetti et al., 1995). Bone marrow examination in patients with concurrent neutropenia often reveals a block of myeloid differentiation at the myelocyte/promyelocyte stage (Benkerrou et al., 1990; Hong et al., 1962; Kyong et al., 1978; Notarangelo et al., 1992). By contrast, serum levels of granulocyte colony-stimulating factor (G-CSF) in neutropenic CD40LG-deficient patients are normal or elevated (Wang et al., 1994).

A European registry of CD40LG-deficient patients has been organized that includes clinical, immunological, and molecular data (Notarangelo et al., 1996). The registry is accessible through at http://bioinf.uta.fi/CD40Lbase. A similar registry has been set up in the United States by USIDnet.

LABORATORY FINDINGS

Immunoglobulin Levels

Like all forms of impaired CSR, CD40LG deficiency is characterized by markedly reduced serum IgG, IgA, and IgE with variable IgM levels and a normal number of circulating B-cells (Geha et al., 1979; Levy et al., 1987; Notarangelo et al., 1992; Winkelstein et al., 2003). Variability of IgM serum levels has been reported among affected members of the same family, indicating that increased IgM may reflect chronic antigenic stimulation rather than the direct effect of a molecular defect (Kroczek et al., 1994). In a series of 56 CD40LG-deficient patients, the majority (53 percent) had normal IgM serum levels at the time of diagnosis (Levy et al., 1997). In another study, however, as many as one quarter of patients with confirmed CD40LG deficiency had low concentrations of serum IgM, indicating that even low serum IgM levels should not preclude testing for CD40LG deficiency (Gilmour et al., 2003). Altogether, the variability of IgM serum levels and the broad range of clinical manifestations, which go beyond abnormalities of humoral immunity, have led researchers to abandon the older designation of "X-linked hyper-IgM" that was previously used to indicate CD40LG deficiency. Although serum levels of IgG, IgA, and IgE are normally very low in patients with CD40LG deficiency, exceptional cases with elevated IgA, IgE, or even IgG have been observed (Levy et al., 1997; L. Notarangelo, unpublished observation), indicating that environmental and/or other genetic factors may have an impact on CSR. In particular, signaling through Toll-like receptors may permit CD40LG/CD40-independent CSR (Glaum et al., 2009; Pasare et al., 2005; Xu et al., 2008).

Serum isohemagglutinins are usually normal (Benkerrou et al., 1990; Kyong et al., 1978; Rosen et al., 1961). In contrast, immunization with T-dependent antigens (e.g., bacteriophage ϕx174) leads to reduced primary and secondary IgM antibody responses and little or no production of IgG-specific antibodies following recall immunization (Benkerrou et al., 1990; Nonoyama et al., 1993; Stiehm and Fudenberg, 1966). In addition, analysis of V_H gene segments in patients with *CD40LG* mutations has revealed a lower frequency of somatic mutations in IgM-expressing B-cells than that in controls (Chu et al., 1995; Razanajaona et al., 1996). This defect is particularly pronounced at the hypermutable G in the RGYW motif, which is a typical target of AICD. Along with the defect in frequency, SHM in patients with CD40LG deficiency is characterized by an increase in transitions versus transversions, reminiscent of decreased UNG activity (Longo et al., 2009). These data are in keeping with the notion that both AICD and UNG are transcriptional targets of CD40LG.

Defects of CSR and SHM are a direct consequence of the underlying genetic defect. Because of the inability to express functional CD40LG trimers, activated T-cells are unable to provide a key helper signal for terminal B-cell differentiation. The functional integrity of their B-cells, by contrast, was initially suggested by the experiments performed by Mayer et al. (1986), who demonstrated that B-cells from these patients can be driven to secrete immunoglobulins of various isotypes in the presence of pokeweed mitogen when co-cultured with "helper T lymphoblasts" from a patient with a Sézary-like syndrome. Subsequently, it was shown that co-culture of patient-derived PBMCs with anti-CD40 mAb (or soluble CD40LG) and appropriate cytokines, such as IL-4 and IL-10, results in normal CSR and in vitro production of IgG, IgA, and IgE

(Allen et al., 1993a; Aruffo et al., 1993; Callard et al., 1994; Durandy et al., 1993; Fuleihan et al., 1993a; Korthauer et al., 1993; Saiki et al., 1995).

B and T Lymphocytes

Subjects with CD40LG deficiency have a normal number of circulating B lymphocytes, and this allows differentiation from X-linked agammaglobulinemia. However, circulating B-cells express IgM and/or IgD, but not other isotypes (Benkerrou et al., 1990; Levitt et al., 1983). Furthermore, since CD40LG–CD40 interaction is essential for memory B-cell generation, the number of circulating $IgD^{-}CD27^{+}$ switched memory B-cells is strongly diminished in patients with CD40LG mutations (Agematsu et al., 1998).

In spite of the T-cell nature of the defect in CD40LG deficiency, the number and distribution of T-cell subsets are normal, although the proportion of $CD45R0^{+}$ primed T-cells is reduced (Jain et al., 1999). In vitro proliferative response to mitogens is normal, but T-cell proliferation to antigens is often reduced (Ameratunga et al., 1997; Levy et al., 1997). A defect in TH1 responses has been reported, with reduced secretion of IFN-γ and failure to induce antigen-presenting cells to synthesize IL-12 (Jain et al., 1999; Subauste et al., 1999). A summary of laboratory features typically observed in CD40LG deficiency is shown in Table 26.1.

MOLECULAR BASIS OF CD40 LIGAND DEFICIENCY

The gene causing X-linked hyper-IgM (as CD40LG deficiency was originally known) was mapped to Xq26.3–27 by linkage analysis (Mensink et al., 1987; Padayachee et al., 1992, 1993). Cloning of the human *CD40LG* gene and coincidental mapping to the same region of the X chromosome (Graf et al., 1992) were soon followed by the recognition that *CD40LG* mutations account for the disease (Allen et al., 1993a; Aruffo et al., 1993; DiSanto et al., 1993; Fuleihan et al., 1993b; Korthauer et al., 1993) and by definition of the gene organization (Shimadzu et al., 1995; Villa et al., 1994). The human *CD40LG* gene encompasses about 13 kb of genomic DNA and is organized in five exons and four introns. Definition of the exon–intron boundaries has

Table 26.1 **SYNOPSIS OF LABORATORY FINDINGS IN PATIENTS WITH CD40LG DEFICIENCY**

PARAMETER	TYPICAL PHENOTYPE	PERCENT OF CD40LG-DEFICIENT PATIENTS SHOWING PHENOTYPE* PHENOTYPE*
Immunological Features		
Serum IgG	<2 SD below normal range	99–100
Serum IgA (mg/dL)	Undetectable	76–93
Serum IgM (mg/dL)	Normal	53–59 (at diagnosis)†
	Elevated	32–47 (at diagnosis) †
Antibody response to T-dependent antigens (φ × 174)	Lack of specific IgG production	100‡
B-cell count	Normal	90
$CD4^{+}$ cell count	Normal	94
$CD8^{+}$ cell count	Normal	98
Proliferative response to PHA	Normal (>50,000 cpm)	93
Proliferative response to T-dependent antigens	Often reduced (<5,000 cpm)	37
CD40LG expression**		
As assessed with CD40-Ig	Absent	Nearly 100††
As assessed with mAb	Usually absent	95
As assessed with polyclonal antiserum	Detectable in some cases	23
Hematological Features		
Neutropenia	Generally present (mostly chronic)	63–68
Anemia	Often present	15–32

* Unless differently specified, data are from Levy et al. (1997) and from Winkelstein et al. (2003).

† Elevated levels of IgM are detected in about 70% of patients during follow-up.

‡ From Nonoyama et al. (1993).

** From a series of 22 patients analyzed at the Department of Pediatrics, University of Brescia, Italy.

†† Activated $CD4^{+}$ T-cells from patients with missense mutations in intracytoplasmic or transmembrane domains of CD40LG may occasionally react with CD40-Ig (Seyama et al. 1998b).

enabled a search for mutations at the genomic level (Lin et al., 1996; Villa et al., 1994).

CD40LG MUTATIONS

Since 1993, a long list of unique *CD40LG* gene mutations have been identified in patients with CD40LG deficiency. Figure 26.2 illustrates the mutations identified in 188 patients included in the European CD40LGbase Registry or the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). Although mutations may affect the entire gene, they are unequally distributed and the majority are located in exon 5, which contains most of the TNF-homology domain (Hollenbaugh et al., 1992).

Missense mutations are the most common cause of the disease. Mutational hot-spots (leading to missense or nonsense mutations) have been identified at codons 140, 155, and 254; none of these involves the presence of CpG dinucleotides. In some cases, for example the Trp140stop mutation, premature termination is compatible with expression of a truncated molecule at the cell surface, as shown by staining with polyclonal anti-CD40LG antibody (Korthauer et al., 1993, Seyama et al., 1998b). Small insertions or deletions in the *CD40LG* gene are also common; these may be due to polymerase slippage, occurring at sites of nucleotide duplications or tandem repeats in the CD40LG sequence (Macchi et al., 1995). A few patients with two different mutations have been described (Aruffo et al., 1993; Grammer et al., 1995; Lin et al., 1996).

Investigation of the effect of amino acid substitutions on CD40LG expression and function, studied through several approaches, has contributed to definition of the role that single residues play in determining folding, assembling, and CD40-binding properties of CD40LG. For some naturally occurring mutations, transfectants that express surface membrane or soluble forms of mutant CD40LG molecules have been generated. The mutagenized recombinants fail to bind CD40-Ig or to induce B-cell proliferation and immunoglobulin secretion in the presence of IL-4 (Allen et al., 1993a; Aruffo et al., 1993).

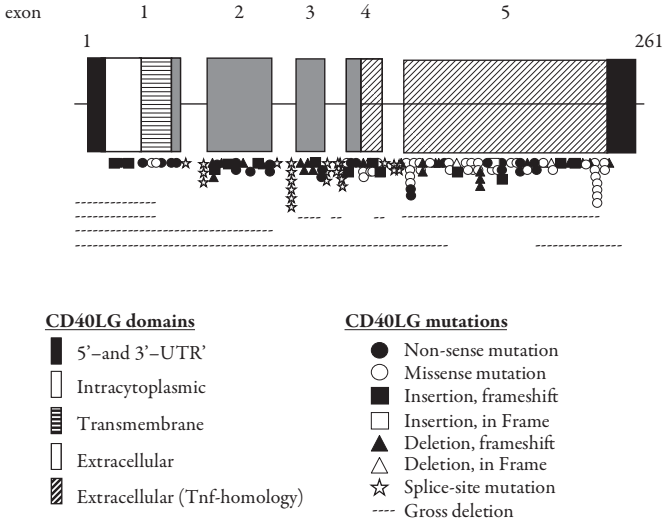

Figure 26.2 Description of *CD40LG* mutations identified in 188 patients from independent families enrolled in the European CD40Lbase Registry or reported to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). The various mutations are shown with different symbols, as indicated in the figure, and aligned along the CD40LG protein. Correspondence between the protein and the five exons of the *CD40LG* gene is also shown.

FUNCTIONAL ASPECTS OF CD40LG DEFICIENCY

Human CD40LG belongs to the TNF family (Hollenbaugh et al., 1992). As mentioned above, mutations cluster predominantly in the extracellular TNF-homology domain. Despite the rather limited sequence identity of the CD40LG TNF-like domain with TNF (27.3 percent), the homology is sufficient to allow computer modeling based on the crystal structures available for TNF and for the TNF/TNFR complex (Banner et al., 1993; Eck and Sprang, 1991; Jones et al., 1989). Thus, models have been generated for murine (Peitsch and Jongeneel, 1993) and human (Bajorath et al., 1995a, 1995b; Notarangelo et al., 1996) CD40LG, and an X-ray structure of the extracellular portion of human CD40LG has been produced (Karpusas et al., 1995). Analogous to TNF, CD40LG forms a trimer and exhibits a remarkably similar overall fold, with the extracellular portion assuming the shape of a truncated pyramid (Karpusas et al., 1995). A disulfide bond between Cys178 and Cys218 stabilizes the top of the molecule. The CD40 binding site consists of a shallow groove formed between two monomers. Buried and solvent-accessible residues of the CD40LG molecule have been identified (Bajorath et al., 1995a, 1995b; Karpusas et al., 1995). Interestingly, several residues (Gly144, Lys143, Tyr145, Arg203, and Gln220) that are predicted to be directly involved in CD40 binding (Bajorath et al., 1995a, 1995b) were found to be mutated in patients with CD40LG deficiency. The crystal structure of the extracellular portion of CD40LG has shown that both hydrophobic and hydrophilic residues form the surface of the CD40 binding site (Karpusas et al., 1995). In addition, a number of buried residues appear to be important for proper monomer folding and trimer formation. By applying structural and bioinformatics tools, the consequences of the missense *CD40LG* mutations have been analyzed in detail in several studies. In one recent study, 40 percent of the missense mutants were predicted to cause major structural abnormalities, and 37 percent affect residues that are important for trimerization or for ligand binding (Thusberg and Vihinen, 2007). In particular, Ser128 and Glu129 (which have both been reported to be mutated) lie close to Lys143 in the three-dimensional structure. Since Lys143 is involved in CD40 binding, it is likely that mutations at codons 128 and 129 disturb ligand–receptor interaction (Bajorath et al., 1996; Karpusas et al., 1995); furthermore, they were shown to cause clashes with other side chains of the molecule (Thusberg and Vihinen, 2007). Similarly, Val126 and Leu 155 (also mutated in patients) participate in the formation of a hydrophobic core that is very close to residues 143–145, which are involved in CD40 binding; it is predicted that these mutations also affect the CD40 binding property of CD40LG, and replacement of Leu155 was also found to have an impact on protein structure.

Finally, three additional mutations (Thr147Asn, Thr211Asp, Gly250Ala) involve residues that are either buried (Thr147) or exposed (Thr211, Gly250) and are located at the interface between monomers; they participate in the formation of three equivalent CD40 binding sites, one per interface between monomers. Mutation at these residues should also affect CD40 binding (Bajorath et al., 1996).

However, the majority of missense mutations reported in patients do not involve the CD40 binding site. The amino acids affected may participate in the generation of the hydrophobic core, so mutations at these residues compromise core packing and folding of the monomer (this is the case for Trp140Arg, Trp140Cys, Trp140Gly, Leu232Ser, Ala235Pro, Val237Glu, Thr254Met, and Leu258Ser) (Bajorath et al., 1996). Alternatively, they may involve buried residues at the interface between monomers; mutations at these sites are likely to disturb trimer formation, as predicted for Ala123Glu, Tyr170Cys, Tyr172His, and Gly227Val (Bajorath et al., 1996; Karpusas et al., 1995). Finally, the amino acid substitutions Met36Arg and Gly38Arg in the transmembrane domain of CD40LG introduce a polar residue in a very hydrophobic sequence, causing severe problems for the insertion of the molecule in the membrane. Indeed, these mutations are associated with a marked reduction in cell surface expression (Garber et al., 1999). By analogy with similar mutations in other proteins, it has been speculated that these mutations promote retention of the mutant in the endoplasmic reticulum and degradation (Thusberg and Vihinen, 2007).

STRATEGY FOR DIAGNOSIS

Along with typical clinical and immunological features that distinguish CD40LG deficiency from XLA, a positive X-linked family history is important in the diagnosis of CD40LG deficiency; however, sporadic occurrence in males is not rare. For this reason, diagnosis of CD40LG deficiency is usually accomplished by demonstrating in vitro the inability of activated patient $CD4^+$ T-cells to express functional CD40LG (CD154) molecules, as assessed by binding of soluble CD40-Ig chimeric constructs or of anti-CD40LG mAbs. However, false-negative results may be obtained with use of mAbs, because occasionally they may recognize mutant forms of CD40LG expressed at the cell surface. This phenomenon is even more common when polyclonal antisera to CD40LG are used in the staining procedure, as they may recognize even truncated forms of the protein (Seyama et al., 1998b). Therefore, polyclonal antisera to CD40LG should not be used for diagnosis. However, rarely even the chimeric CD40-Ig molecule may bind to mutant CD40LG, particularly in patients with missense mutations in the transmembrane or cytoplasmic domains, which are permissive for membrane protein expression (Lee et al., 2005; Seyama et al., 1998a, 1998b). Ultimately, mutation analysis at the *CD40LG* locus may be required for a definitive diagnosis.

A number of critical factors should be considered when performing diagnostic assays for CD40LG deficiency. First, appropriate controls for T-cell activation (e.g., expression of CD69) should be included. This is particularly important to distinguish CD40LG deficiency from common variable immunodeficiencies (CVIDs); a subgroup of CVIDs patients have defective CD40LG expression in the context of a broader T-cell activation defect (Farrington et al., 1994). Second, because CD40LG is preferentially expressed by activated $CD4^+$ cells (Lane et al., 1992), any condition characterized by CD4 lymphopenia would also result in a low proportion of CD40LG-expressing cells upon activation in vitro. In particular, it has been recognized that activated T-cells from patients with MHC class II deficiency fail to express CD40LG because of the markedly reduced proportion of $CD4^+$ T lymphocytes (Callard et al., 1994). Third, the age of the proband has to be considered. There is ample evidence showing that expression of CD40LG by activated neonatal T-cells is physiologically reduced (Brugnoni et al., 1994; Durandy et al., 1995; Fuleihan et al., 1994; Nonoyama et al., 1995). As mentioned above, definitive confirmation of CD40LG deficiency can be achieved through *CD40LG* gene mutation analysis.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

In contrast to carriers of other X-linked immunodeficiencies (e.g., X-linked agammaglobulinemia [XLA]; X-linked severe combined immunodeficiency [X-SCID]; Wiskott-Aldrich syndrome [WAS]), carrier females of CD40LG deficiency exhibit a random pattern of X inactivation and are indeed mosaics for two populations of circulating T lymphocytes (one expressing the wild-type CD40LG allele and the other expressing the mutated one) (Hollenbaugh et al., 1994). In carriers with skewed lyonization, no clinical or immunological abnormalities are found, indicating that limited CD40LG expression is sufficient to induce isotype switching and normal generation of memory B-cells (Callard et al., 1994; Hollenbaugh et al., 1994). However, extreme lyonization with selective expression of the mutant form of CD40LG may exceptionally result in an overt clinical phenotype (de Saint Basile et al., 1999).

Carrier detection is best achieved with *CD40LG* gene molecular analysis. In families with clear X-linked inheritance, linkage analysis can be performed, taking advantage of two sets of hypervariable microsatellites at the 3′ untranslated region of the *CD40LG* gene (Allen et al., 1993b; DiSanto et al., 1993, 1994; Gauchat et al., 1993a; Ramesh et al., 1994; Shimadzu et al., 1995). Whenever the mutation is known, DNA sequencing, with a search for heterozygosity for the specific mutation, is the most simple and direct way to attempt carrier detection (Lin et al., 1996; Shimadzu et al., 1995; Villa et al., 1994). Heterozygosity for the mutation (e.g., carrier detection) can also be investigated at the cDNA level, as for splice-site mutations that cause exon skipping (DiSanto et al., 1993; Hollenbaugh et al., 1994).

Knowledge of the specific *CD40LG* gene defect also allows prenatal diagnosis on chorionic villi DNA by 10 to 11 weeks of gestation (Villa et al., 1994). Segregation analysis with hypervariable microsatellites at the 3′ untranslated region of the *CD40LG* gene has been also successfully used for this purpose (DiSanto et al., 1994).

Despite the reduced ability of activated T-cells from neonates to express CD40LG (Brugnoni et al., 1994; Durandy et al., 1995; Fuleihan et al., 1994; Nonoyama et al., 1995), surface membrane CD40LG was detected on activated T-cells from 19- to 28-week-old fetuses (Durandy et al., 1995). However, because of the obviously complex developmental control of CD40LG expression, staining for CD40LG on fetal cord blood T lymphocytes should not be used as the sole technique for prenatal diagnosis of CD40LG deficiency.

TREATMENT AND PROGNOSIS

The long-term prognosis of CD40LG deficiency appears to be worse than that in other forms of congenital hypogammaglobulinemia—for example, XLA. Data from the European registry on 128 patients with CD40LG deficiency indicated that survival at 25 years of age was only 40 percent. In addition, 30 of the 128 patients had died. Mortality was somewhat lower in the U.S. registry, in which 8 of 79 subjects had died. This difference in mortality between the European and the U.S. series may reflect a lower incidence of *Cryptosporidium* infection, sclerosing cholangitis, and irreversible liver damage in the latter (Winkelstein et al., 2003). The main causes of death in patients with CD40LG deficiency include infections early in life and, later on, severe liver disease and malignant tumors (Hayward et al., 1997; Levy et al., 1997).

The complex array of clinical manifestations and the increased risk of opportunistic infections and chronic neutropenia require multiple therapeutic approaches. Regular infusion of IVIG (400–600 mg/kg every 21–28 days) is the most important form of treatment. It significantly reduces the severity and frequency of infections (Levy et al., 1997) and may occasionally correct neutropenia (Banatvala et al., 1994; Levy et al., 1997). In addition, prophylaxis with co-trimoxazole is necessary to prevent *P. jiroveci* pneumonia (Banatvala et al., 1994; Levy et al., 1997; Notarangelo et al., 1992). Long-term treatment with amphotericin B and flucytosine has been used in patients who have developed cryptococcosis (Iseki et al., 1994; Tabone et al., 1994). Patients with severe neutropenia may benefit from treatment with recombinant G-CSF; in some cases, this approach has caused a change from chronic to cyclic neutropenia (Shimadzu et al., 1995; Wang et al., 1994). Total parenteral nutrition may be necessary in patients with protracted diarrhea and malabsorption, particularly if these are due to *Cryptosporidium* (Benkerrou et al., 1990). *Cryptosporidium* infection should be prevented by avoiding risk factors (such as swimming in lakes), but use of filtered or sterile water at home has been also suggested. Early detection of *Cryptosporidium* infection is best achieved by PCR-based amplification of stool DNA and microscopy of bile fluid (McLauchlin et al., 2003). Acute infection has been treated with azithromycin or nitazoxanide, but results have been often unsatisfactory.

Because of the rather dismal prognosis, more radical forms of treatment have been proposed, in particular hematopoietic cell transplantation (HCT), which at the moment remains the only strategy that can result in permanent cure of the disease (Thomas et al., 1995). In a series of 38 European CD40LG-deficient patients treated by HCT, 58 percent were cured; among those without preexisting liver disease the survival rate was 72 percent. However, the overall mortality rate was high (31.6 percent) (Gennery et al., 2004), and in all cases, death was from infections. Preexisting lung disease was associated with a poor outcome. Promising results have been obtained using a nonmyeloablative conditioning regimen in CD40LG-deficient patients with severe liver disease (Jacobsohn et al., 2004; Kikuta et al., 2006). Importantly, lack of a strict genotype–phenotype correlation in CD40LG deficiency prevents selection of high-risk patients who might benefit from HCT early in life.

Attempts to treat severe liver disease (sclerosing cholangitis, cirrhosis) with liver transplantation usually fail because of relapse of the disease in the transplanted organ (Hayward et al., 1997; Levy et al., 1997). Combined bone marrow and cadaveric orthotopic liver transplantation has resulted in disease correction in one patient (Hadzic et al., 2000); however, additional severely affected CD40LG-deficient patients treated with HCT and liver transplantation have failed to survive (Notarangelo, unpublished).

The recognition that expression of the *CD40LG* gene is under tight regulatory control makes gene therapy a less viable option, particularly since deregulated expression of CD40LG in transgenic mice has been shown to result in tumor development (Brown et al., 1998; Sacco et al., 2000). Use of lentiviral vectors might enable insertion of autologous regulatory gene elements (Barry et al., 2000), thus making gene therapy–based treatment with acceptable risks possible in the future. However, the finding that mutant forms of CD40LG interact with wild-type molecules and prevent expression of functional trimers (Seyama et al., 1999; Su et al., 2001) raises further doubts that gene therapy could become an effective form of treatment for CD40LG deficiency. These problems may be circumvented by *trans*-splicing (a process by which two different pre-mRNAs are joined by the cellular splicing apparatus), which allows complementation of the gene defect while preserving the natural regulation and cell specificity of CD40LG expression. Indeed, this strategy has been successfully used to correct a murine model of CD40LG deficiency (Tahara et al., 2004).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF CD40 DEFICIENCY

Defective expression of CD40 by B lymphocytes (CD40 deficiency, MIM *606843) has been detected in five children from four unrelated families from the Mediterranean region (Ferrari et al., 2001; Kutukculer et al., 2003; Lougaris et al., 2005; Mazzolari et al., 2007). Twelve additional patients with molecularly proven CD40 deficiency have been identified in Saudi Arabia (El-Ghonaium, personal communication). Clinical and immunological data at presentation were very similar to those observed in CD40LG deficiency, but autosomal recessive inheritance was indicated by parental consanguinity and by the fact that four of the five reported patients were female. Patient 1, an 8-year-old Italian girl, suffered from

P. jiroveci pneumonia at 4 months of age and had another episode of pneumonia at age 2, when she was found to be panhypogammaglobulinemic and was started on IVIG treatment (Lougaris et al., 2005). At the age of 7 years, she developed persistent eosinophilia (800–13,500/μL) and a mild increase of liver enzymes. Liver biopsy showed a severe pattern of sclerosing cholangitis, and microscopic examination of bile specimens revealed the presence of *Cryptosporidium* oocysts. The patient died at the age of 9 years from liver insufficiency. Patients 2 and 3 are first cousins from a multiply related Arabian family. Patient 2 is a 5-year-old boy who suffered from recurrent pneumonia, hypogammaglobulinemia with elevated IgM, and neutropenia. Patient 3 is a 7-year-old girl who also experienced recurrent lower respiratory tract infections. She was diagnosed with immunodeficiency with hyper-IgM at 8 months of age, and substitution treatment with IVIG was started. At 3 years of age, she was admitted to a pediatric intensive care unit for severe interstitial pneumonia. Patient 4, a 12-month-old Turkish girl born to consanguineous parents, was hospitalized for respiratory distress. She developed necrotizing pneumonia caused by *Pseudomonas aeruginosa* and chronic watery diarrhea and disseminated *Cryptosporidium parvum* infection. She received a matched-sibling stem cell transplantation but died of cardiorespiratory arrest at day 16 posttransplant. Patient 5 was hospitalized at 2 years of age because of recurrent pneumonia, otitis, and skin infections and was diagnosed with a hyper-IgM phenotype associated with impaired CD40 expression. She developed neutropenia that required treatment with G-CSF. At 3 years of age, she received HCT from her HLA-identical healthy carrier sibling. She is now fully reconstituted at 4 years after HCT.

All five children had very low levels of IgG and IgA, and three of them had increased serum IgM levels. Lymphocyte numbers and subset distributions were normal, as were in vitro proliferative responses to mitogens. However, CD40 expression on the surface of B lymphocytes and monocytes was abrogated or severely reduced. Importantly, in contrast to what observed in CD40LG deficiency, B-cells from CD40-deficient patients could not be induced to secrete IgG and IgA upon in vitro activation with anti-CD40 mAb and IL-10, indicating a B-cell intrinsic defect.

MOLECULAR AND IMMUNOLOGICAL FEATURES OF CD40 DEFICIENCY

Patients with CD40 deficiency are clinically and immunologically undistinguishable from those with CD40LG deficiency. However, circulating B-cells and monocytes lack surface membrane CD40, whereas expression of CD40LG on the surface of in vitro activated $CD4^+$ T-cells is preserved. Western-blot analysis of lymphoblastoid cell lines showed that the CD40 protein was also undetectable intracellularly in patient 1, whereas an aberrant pattern of migration of the CD40 protein was detected in patients 2, 4, and 5. Mutation analysis at the *CD40* gene showed that all of the patients carried homozygous mutations (Fig. 26.3). In particular, patient 1 was homozygous for a silent mutation (A-to-T substitution at nucleotide 408, corresponding to the fifth nucleotide of exon 5), which involves and disrupts an exonic splicing enhancer, thus preventing incorporation of exon 5 in the mRNA. Consequently, cDNA from this patient lacked 94 nucleotides (matching exon 5), resulting in frameshift and premature termination. Patients 2 and 3 were both homozygous for a C-to-T change at nucleotide 247, resulting in a nonconserved Cys83-to-Arg amino acid substitution. The mutation in patient 4 occurred at position 2 of the acceptor site of intron 3; use of a cryptic splice site in this patient resulted in a 2-a.a. deletion (ΔN86_L87) and replacement of the next a.a. (G88R). Patient 5 was homozygous for a 3-nucleotide deletion in exon 2, resulting in one a.a. deletion (del I33) in the extracellular domain of CD40. This was also the only patient who had residual, though markedly reduced, CD40 expression at the cell surface.

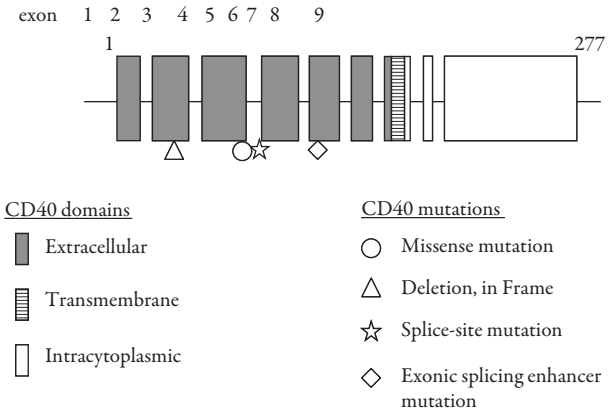

Figure 26.3 Description of *CD40* mutations in affected individuals from four unrelated families with CD40 deficiency. The different types of mutations identified are indicated with different symbols and are aligned along the structure of the CD40 protein with its extracellular, transmembrane, and intracytoplasmic domains. Correspondence between the protein and the nine exons of the gene is also shown.

Investigation of the intracellular fate and biochemical properties of CD40 mutants carrying amino acid substitutions has revealed that the mutant proteins are synthesized but retained in the endoplasmic reticulum (ER). Moreover, accumulation of the C83R is associated with ER stress and activation of the unfolded protein response. By contrast, the ΔN86_L87;G88R mutant is efficiently disposed of by the ER-degradation pathway, whereas the ΔI33 partially negotiates transport to the cell membrane and is competent for CD40LG binding and intracellular signaling (Lanzi et al., 2010). Further investigation of the functional consequences of CD40 deficiency showed that both memory B-cell generation and somatic mutation were affected. DCs, cultured with TNF-α or with lipopolysaccharide (LPS) combined with IFN-γ, displayed a consistent defect in their ability to induce proliferation of allogeneic T-cells and secretion of IFN-γ. The defective co-stimulatory activity of DCs derived from patients with CD40 deficiency was associated with lower cell surface levels of MHC class II antigen and with a decreased release of IL-12. These findings support the notion that CD40 deficiency is not an exclusive defect of humoral immunity but should be considered a combined defect of B- and T-cell compartments (Fontana et al., 2003).

The diagnosis of CD40 deficiency is usually made by demonstrating the inability of peripheral blood B-cells to express CD40, as assessed by flow cytometry, in patients with clinical features and an immunoglobulin profile suggestive of impaired CSR. However, the possibility of false-negative results, due to recognition of mutant CD40 molecules by the monoclonal antibody, exists. Furthermore, because CD40 is expressed as a trimer, is it theoretically possible that heterozygous mutations that allow expression of CD40 but affect its function may result in an HIGM phenotype through a dominant-negative effect. Ultimately, diagnosis of CD40 deficiency requires mutation analysis at the *CD40* locus.

TREATMENT AND PROGNOSIS

Management of patients with CD40 deficiency is similar to that outlined for patients with CD40LG deficiency. Treatment includes regular infusions of IVIG, *Pneumocystis* pneumonia prophylaxis with co-trimoxazole, measures to prevent *Cryptosporidium* infection, and monitoring of liver status by ultrasound scanning and biochemical analysis. A persistent increase of liver enzymes and/or eosinophils requires a careful investigation for the presence of sclerosing cholangitis and/or *Cryptosporidium* infection, as these events seem to be highly correlated. For this purpose, microscopic examination of the stools may not be sensitive enough, and more sensitive methods should be applied, such as PCR-based amplification of stool DNA and microscopy of bile fluid. Although CD40 expression is not restricted to hematopoietic cells, successful experience with HCT in one patient with CD40 deficiency indicates that correction of the defect on hematopoietic cells is sufficient to cure the disease.

ANIMALS MODELS OF CD40 AND CD40LG DEFICIENCY

GENE DISRUPTION

Mice with Disruption of the *CD40LG* Gene

Cd40lg$^{-/-}$ mice exhibit normal percentages of B- and T-cell subpopulations but display selective deficiencies in humoral immunity. Basal serum immunoglobulin isotype levels are significantly lower than in normal mice, and IgE is undetectable. Furthermore, *cd40lg*$^{-/-}$ mice fail to mount secondary antigen-specific responses to immunization with T-dependent antigens. By contrast, they produce antigen-specific antibody of all isotypes except IgE in response to thymus-independent antigens. These results underscore the requirement of CD40LG for T-cell–dependent antibody responses (Renshaw et al., 1994; Xu et al., 1994). Moreover, Ig class switching to isotypes other than IgE can occur in vivo in the absence of CD40LG, a phenomenon supporting the notion that alternative B-cell signaling pathways (such as TLR-mediated B-cell activation) regulate responses to thymus-independent antigens.

CD4^{+} T-cells from *cd40lg*$^{-/-}$ mice were fourfold less effective than normal T-cells in activating the nitric oxide response in allogeneic macrophages. *Cd40lg*$^{-/-}$ T-cells fixed with paraformaldehyde after a 6-hour activation period, a time point at which CD40LG dominates the macrophage-activating capability of T-cells, failed to activate the production of inflammatory cytokines (TNF-α) or the generation of reactive nitrogen intermediates. After 24 hours of activation, however, both *Cd40lg*$^{-/-}$ and normal T-cells could induce similar but weak responses from activated macrophages (Stout et al., 1996). These studies demonstrate that *Cd40lg*$^{-/-}$ mice have a deficient T-cell–dependent macrophage-mediated immune response. However, *Cd40lg*$^{-/-}$ mice are able to generate normal primary cytotoxic T-cell responses (in spite of a defective humoral response) to a viral infection (Whitmire et al., 1996).

Cd40lg$^{-/-}$ mice are susceptible to *P. jiroveci* infection (as are CD40$^{-/-}$ mice). Treatment of wild-type mice with soluble CD40LG-fusion protein evokes a pulmonary inflammatory response that is not observed in identically treated *Cd40*$^{-/-}$ mice (Wiley et al., 1997). This finding supports evidence that ligation of CD40 results in inflammatory responses and that soluble CD40LG is a potent inflammagen that may be important for protection against *P. jiroveci* infection.

Finally, when injected with *Cryptococcus neoformans*, *Cd40lg*$^{-/-}$ mice show increased fungal growth in the brain, associated with reduced production of IL-12, IFN-γ, and nitrites (Pietrella et al., 2004).

MICE WITH DISRUPTION OF THE *Cd40* GENE

Cd40$^{-/-}$ mice have normal numbers of T and B-cells, indicating that CD40 is not essential for B-cell development. Their B-cells fail to proliferate and undergo isotype switching in response to soluble CD40 ligand (sCD40LG) and IL-4 but respond normally to LPS in the presence of IL-4. *Cd40*$^{-/-}$ mice completely fail to mount an antigen-specific antibody response or to develop germinal centers following immunization with T-cell–dependent antigens, but they respond normally to the T-cell–independent antigens. The most noticeable alteration in the serum immunoglobulin levels of young *Cd40*$^{-/-}$ animals is absence of IgE and a severe decrease of IgG1 and IgG2a (Castigli et al., 1994; Kawabe et al., 1994). These results indicate an essential role of CD40–CD40LG interactions in the antibody response to T-cell–dependent antigens and in isotype switching.

B-cells deficient in CD40 expression are unable to elicit the proliferation of allogeneic T-cells in vitro. More importantly, mice immunized with *Cd40*$^{-/-}$ B-cells become tolerant to allogeneic MHC antigens as measured by a mixed lymphocyte reaction and cytotoxic T-cell assay. The failure of *Cd40*$^{-/-}$ B-cells to serve as antigen-presenting cells in vitro is corrected by the addition of anti-CD28 mAb. Moreover, LPS stimulation, which upregulates CD80/CD86 expression, reverses the inability of *Cd40*$^{-/-}$ B-cells to stimulate an alloresponse in vitro and abrogates the capacity of these B-cells to induce tolerance in vivo (Hollander et al., 1996). These results suggest that CD40 engagement by CD40 ligand expressed on antigen-activated T-cells is critical for the upregulation of

CD80/CD86 molecules on antigen-presenting B-cells that subsequently deliver the co-stimulatory signals necessary for T-cell proliferation and differentiation.

In addition to susceptibility to *P. jiroveci* infection, CD40-deficient mice are also prone to *Mycobacterium avium* infection (Florido et al., 2004). Furthermore, when inoculated with the defective murine leukemia retrovirus LP-BM5def, *Cd40*$^{-/-}$ mice become infected and show virus expression similar to that in wild-type mice. However, unlike the wild-type mice, CD40-deficient mice do not develop symptoms of immunodeficiency, lymphoproliferation, and the typical histological changes in the lymphoid tissue (Yu et al., 1999). These results show that the CD40–CD40LG interaction in vivo is essential for anergy induction and the subsequent development of immunodeficiency and pathological expansion of lymphocytes.

In keeping with similar in vitro observations, mice deficient in CD40LG or in CD40 expression show impaired cross-talk between activated T-cells and DCs, with failure to expand IL-17–producing cells, and are resistant to the development of experimental autoimmune encephalitis (Iezzi et al., 2009).

Cd40$^{-/-}$ adult mice develop neuronal cell dysfunction and gross central nervous system abnormalities with age. These findings suggest that CD40 signaling plays an important role in normal neuronal cell maintenance and confers resistance to aging-induced stress (Tan et al., 2002).

CONCLUSION

Detailed clinical, molecular, and immunological analysis of patients with CD40LG or with CD40 deficiency has been instrumental in unraveling the complex effects that CD40LG (CD154) exerts in vivo upon interaction with CD40, and that extend beyond promoting B-cell activation and differentiation. The broad range of cellular effects that CD40LG/CD40 interaction promotes also account for the complexity of immune defects that characterize deficiency of CD40LG or of CD40. Accordingly, these disorders are currently classified among human combined immunodeficiencies (Notarangelo et al., 2009), and use of more aggressive forms of treatment (such as HCT) than immunoglobulin replacement therapy alone has been advocated. At the same time, involvement of CD40LG/CD40 signaling in inflammation and in tumor cell killing has prompted use of biological modifiers that interfere with the CD40-signaling pathway in autoimmune, inflammatory, and neoplastic diseases and in prevention of graft rejection and graft-versus-host disease (Durie et al., 1994).

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27.

AUTOSOMAL Ig CSR DEFICIENCIES CAUSED BY AN INTRINSIC B-CELL DEFECT

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The study of inherited hyper-IgM syndromes (Ig CSR deficiency) has greatly contributed to our understanding of the normal processes of antibody maturation because these syndromes have in common a defect in immunoglobulin (Ig) class-switch recombination (CSR), as demonstrated by normal or elevated serum IgM levels. This is in contrast to absent or strongly decreased levels of the other Ig isotypes. Antibody maturation leads to the production of antibodies of different isotypes and formation of B-cell receptors (BCRs) with high affinity for antigen. This event usually takes place in the secondary lymphoid organs (spleen, lymph nodes, tonsils) in an antigen- and T-lymphocyte–dependent manner. When mature (but still naïve) IgM^+IgD^+ B cells, after emigrating from the bone marrow (or fetal liver), encounter an antigen that is specifically recognized by their BCRs, they proliferate vigorously and give birth to a unique lymphoid formation, the germinal center. In this location, B cells undergo the two major events of maturation: CSR and somatic hypermutation (SHM).

CSR AND SHM

CSR is a process of DNA recombination between two different switch (S) regions located upstream of the constant (C) regions, while the intervening DNA is deleted by forming excision circles (Iwasato et al., 1990; Kinoshita and Honjo, 2000; Manis et al., 2002; Matsuoka et al., 1990; von Schwedler et al., 1990). Replacement of the Cμ region by a Cx region from another class of Ig results in the production of antibodies of different isotypes (IgG, IgA, and IgE) with the same variable (V) region and thus the same antigen specificity and affinity. The different Ig isotypes vary in activities (half-life, binding to Fc receptors, ability to activate the complement system) and tissue localization (IgA is secreted by mucosal membranes). Thus CSR is necessary for an optimal humoral response against pathogens.

Through SHM, missense mutations and, less frequently, deletions or insertions are introduced into the V regions of immunoglobulins. This process is triggered by activation of the BCR and signaling via CD40 (Jacobs et al., 2001; Storb et al., 1998). These mutations occur at a high frequency in the V regions and their proximal flanks (1×10^3 bases/generation). SHM is required as a basis for the selection and proliferation of B cells expressing a BCR with a high affinity for antigen in close interaction with follicular dendritic cells (Frazer et al., 1997; Rajewsky, 1996). The CSR and SHM processes occur simultaneously in germinal centers, but neither is a prerequisite for the other because IgM may be mutated whereas IgG or IgA can remain unmutated (Jacob and Kelsoe, 1992; Kaartinen et al., 1983).

Three successive steps are required for the process of CSR and SHM:

1. Transcription of the targeted DNA (S and V regions). In S regions, this step leads to the formation of RNA–DNA hybrids, known as stable R-loops, on the template DNA strand, leaving the single nontemplate strand accessible for cleavage (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003; Yu et al., 2003).
2. DNA cleavage. During CSR, single-stranded DNA breaks result in double-stranded DNA breaks (DSBs) by a mechanism currently unknown. It has been suggested that the template DNA strand may also be attacked in transcription bubbles (Bransteitter et al., 2003), resulting in scattered DSBs, eventually processed by exonucleases and error-prone polymerases into blunt DSBs. This

error-prone DNA processing is suggested by the high frequency of mutations found in Sμ–Sx junctions (Chen et al., 2001). In V regions, single-stranded DNA breaks are either directly repaired or processed into staggered DSBs. Alternatively, recent data suggest that blunt DSBs could occur spontaneously in V regions and secondarily lead to staggered DSBs (Catalan et al., 2003; Zan et al., 2003).

3. DNA repair. The mechanisms involved in DNA repair differ for CSR and SHM. During CSR, histone H2AX is phosphorylated, and the repair protein 53BP1 and the complex MRE11/RAD50/NBS1 are recruited at DSBs in repair foci. Thereafter, the DNA repair machinery joins Sμ and Sx sequences by means of the widespread, constitutively expressed nonhomologous end-joining (NHEJ) enzymes, including the KU proteins that act on DNA blunt DSBs (Casellas et al., 1998; Manis et al., 1998; Rolink et al., 1996). In contrast, SHM DNA repair does not require the NHEJ complex (Bemark et al., 2000), but probably the error-prone polymerases η and the mismatch repair (MMR) enzymes (Cascalho et al., 1998; Evans and Alani, 2000; Kenter, 1999; Schrader et al., 1999; Wiesendanger et al., 1998).

CSR and SHM are initiated by T- and B-cell interaction, involving CD40 ligand (CD40L or CD154), a molecule transiently expressed on activated $CD4^+$ T cells, and CD40, constitutively expressed on B lymphocytes. The CD40L–CD40 interaction is required for B-cell proliferation, germinal-center formation, CSR, and SHM, as demonstrated by the phenotype of patients with loss of function of either the *CD40L* (Aruffo et al., 1993; DiSanto et al., 1993) or *CD40* gene (Ferrari et al., 2001). The role of the CD40 activation pathway in antibody maturation has been underscored by the recent demonstration of a profound immune deficiency, including the Ig CSR deficiency phenotype, as the result of defective nuclear factor-κB (NF-κB) signaling following CD40 activation. This is illustrated by the fact that hypomorphic mutations in the zinc finger domain of the NF-κB essential modulator gene (also called *NEMO* or *IKKγ*) (Döffinger et al., 2001; Jain et al., 2001; Zonana et al., 2000) or mutation producing a gain of function of the NF-κB inhibitor IκBα (Courtois et al., 2003) result, respectively, in a syndrome of X-linked or autosomal dominant anhidrotic ectodermal dysplasia (AED) associated with a T-cell immunodeficiency and the Ig CSR deficiency condition.

Ig CSR deficiency syndromes with an autosomal recessive mode of inheritance have been described over the years by a number of investigators (Callard et al., 1994; Conley et al., 1994; Durandy et al., 1997). This condition is characterized by a specific B-cell defect, resulting in increased susceptibility to bacterial infections (but not to opportunistic infections) that can be easily controlled by regular intravenous Ig (IVIG) substitution. Lymphadenopathy is frequent (50–75 percent) and autoimmunity is reported in 25 percent of patients. SHM is found to be either normal or defective, according to the molecular defect. B cells are intrinsically defective; although they normally proliferate, they are unable to undergo CSR after activation by CD40L and cytokines. The precise delineation of the defects leading to abnormal CSR allowed the definition of molecularly defined autosomal recessive Ig CSR deficiency conditions.

ACTIVATION-INDUCED CYTIDINE DEAMINASE DEFICIENCY

The activation-induced cytidine deaminase (*AICDA*) gene (*605257) was first identified in mice (Muramatsu et al., 1999) and cloned by substractive hybridization between murine lymphoma CH12F-2 B cells with and without induction of CSR in vitro. The *AICDA* RNA transcripts are detected only in B cells undergoing CSR or SHM either in vivo (in germinal-center B cells) or in vitro (Diaz and Casali, 2002; Faili et al., 2002; Muramatsu et al., 1999; Papavasiliou and Schatz, 2002; Revy et al., 2000). The activation-induced cytidine deaminase (AID) protein is structurally similar (34 percent amino acid sequence identity) to the apoB mRNA-editing enzyme APOBEC-1. RNA editing is widely used to create new functional RNAs from a single gene. APOBEC-1 edits ApoB mRNA by deamination of a cytosine into a uracil residue at a specific site, resulting in a stop codon. ApoB 100 and ApoB48, the translation products of the unedited and edited apoB mRNAs, respectively, have entirely different functions and expression profiles (Mehta et al., 2000; Navaratnam et al., 1993; Teng et al., 1993). APOBEC-1 requires an auxiliary factor (ACF) for the site-specific editing of apoB mRNA. This auxiliary factor is widely expressed, including in tissues that do not express APOBEC-1 (Navaratnam et al., 1993; Teng et al., 1993; Yamanaka et al., 1994).

The open reading frame of the *AICDA* cDNA encodes a 198-residue protein with a molecular mass of approximately 24 kDa. This protein contains an active site for cytidine deamination, whose sequence is conserved throughout the large cytidine deaminase family, and has been shown to display cytidine deaminase activity *in vitro* (Muramatsu et al., 1999). The C-terminal domain also contains a leucine-rich region that may be important for protein–protein interaction. This region is thought to bind accessory factors required for AID activity and may be important in AID tetramerization (Dickerson et al., 2003). Recently, a nuclear localization signal (NLS) and a nuclear export signal (NES) have been described in the N and C termini of the molecule (Ito et al., 2004), although the function of this NLS is still debated (Brar et al., 2004; McBride et al., 2004). An APOBEC-1-like domain is also described but its function remains unknown (Fig. 27.1).

AID-deficient patients and $AID^{-/-}$ mice display a defect in both CSR and SHM, which demonstrates the crucial role of AID in both these processes required for B-cell terminal differentiation. The mechanism of action of AID, however, remains open to debate. Because the sequence of AID is similar to that of the RNA-editing enzyme APOBEC-1, originally it was proposed that AID edits an mRNA encoding a substrate common to CSR and SHM, probably an endonuclease (Chen et al., 2001; Honjo et al., 2002; Kinoshita and Honjo,

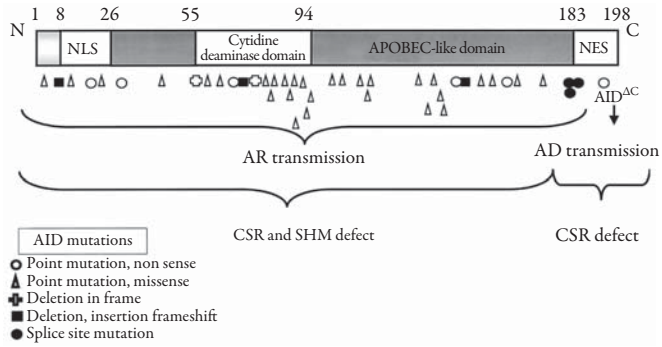

Figure 27.1 Schematic representation of activation-induced cytidine deaminase (AID) and localization of mutations in AID deficiency. Mutations are scattered throughout the entire gene. Each symbol represents a unique mutation. Mutations in the C-terminal domain do not affect somatic hypermutation (SHM). A mutation of the C-terminal region ($AID^{\Delta C}$) results in truncation of the last nine amino acids of the nuclear export signal (NES) and is responsible for an autosomal dominant (AD) form of Ig CSR deficiency. AR, autosomal recessive; CSR, class-switch recombination; NLS, nuclear localization signal; AD, autosomal dominant.

2001). The recently described requirement for de novo protein synthesis downstream of AID expression in CSR, compatible with the synthesis of a recombinase, is consistent with the RNA-editing model (Doi et al., 2003).

However, recent data strongly indicate that AID exerts a DNA-editing activity. Following the transfection of *Escherichia coli*, AID deaminates deoxycytidine (dC) residues within DNA into deoxyuridine (dU) (Petersen-Mahrt et al., 2002). Subsequently, several groups have demonstrated in cell-free assays a direct role for AID on single-stranded DNA but not on double-stranded DNA, RNA–DNA hybrids, or RNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003). The transcription of S regions increases AID activity (Ramiro et al., 2003; Shinkura et al., 2003), probably by generating the secondary structures required for this activity (Yu et al., 2003). R-loops are generated by the formation of RNA–DNA hybrids on the template DNA strand, rendering the single nontemplate strand a target for AID to generate the first lesion required for DNA cleavage. Although these observations provide strong evidence that AID has DNA-editing activity, they were obtained in nonphysiological conditions (overexpression in *E. coli*, using in vitro assays) in which the well-known RNA-editing protein APOBEC-1 exerts a similar effect (Harris et al., 2002; Petersen-Mahrt and Neuberger, 2003).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF AID DEFICIENCY

Four reports describing AID deficiency (MIM #605258) have been published in the literature (Lee et al., 2005; Minegishi et al., 2000; Quartier et al., 2004; Revy et al., 2000). Although the onset of symptoms occurs during early childhood (mean age, 5 years), the diagnosis is frequently established at a later age.

According to clinical data from 51 AID-deficient patients, all presented with recurrent bacterial infections; more than half (58 percent) had respiratory tract infections, with 14 percent of the patients reporting bronchiectasis. Gastrointestinal infections were observed in 27 percent of cases, sometimes related to persistent *Giardia* infections. Such infections may result in failure to thrive. Infections of the central nervous system (e.g., meningitis) have been reported in 25 percent of AID-deficient patients, often associated with inadequate Ig substitution. One case each of herpes virus encephalitis and poliomyelitis have been described. Two adult patients died prematurely, one of pulmonary hemorrhage at 47 years of age, the other of septicemia at age 63.

A striking lymphoid hyperplasia is present in the majority (75 percent) of patients, affecting predominantly the cervical lymph nodes and tonsils. In one case, mesenteric lymph node hyperplasia resulted in intestinal obstruction. Hepatosplenomegaly has been reported in 10 percent of the patient cohort. Other manifestations include arthritis (12 percent) and autoimmune manifestations (hemolytic anemia, thrombopenia, and autoimmune hepatitis) (29 percent); autoantibodies of the IgM isotypes are detectable in some cases. Systematic lupus erythematosus, diabetes mellitus, and Crohn's disease have been reported in one patient each (Table 27.1).

LABORATORY FINDINGS IN AID DEFICIENCY

All patients had normal or elevated IgM at the time of diagnosis and markedly diminished or, most often, undetectable serum levels of IgG and IgA. In some patients, 7S IgM can be demonstrated. In agreement with the aberrant serum immunoglobulin levels, antigen-specific (vaccine or infectious agents) antibodies of the IgG isotype were not detectable. When analyzed, IgM iso-hemagglutinins and antipolysaccharide IgM antibodies were present. IgM serum levels often diminish after Ig substitution, a finding suggesting that increased IgM reflects chronic antigenic stimulation rather than a direct effect of the AID deficiency.

Numbers of peripheral blood T cells ($CD3^+$) and T-cell subsets ($CD4^+$ and $CD8^+$) as well as in vitro T-cell proliferation to mitogens and antigens are normal. Peripheral blood B cells ($CD19^+$) are normal in number mostly and express wild-type CD40 in normal concentrations. All $CD19^+$ B cells co-express sIgM and sIgD, in contrast to age-matched controls, who have a population of $CD19^+$ B cells that express sIgG or sIgA and not sIgM/sIgD.

Soluble CD40-L (sCD40L)-induced B-cell proliferation in vitro is normal. However, in vitro activation of B lymphocytes by sCD40L and interleukin (IL)-4, which induces IgE production in controls and CD40L-deficient patients (Durandy et al., 1993), is ineffective in AID-deficient patients. Under the same culture conditions, S-region transcription is normally induced, whereas DNA DSBs are not detected in Sμ regions, providing evidence that the CSR defect is located downstream from transcription and upstream from DNA cleavage (Catalan et al., 2003). A normal fraction (20–50 percent) of B cells express the CD27 marker, which indicates memory B cells, even if limited to the IgM^+/IgD^+ B-cell compartment (Klein et al., 1998). However, the frequency of

Table 27.1 CLINICAL FEATURES OF CSR-DS CAUSED BY AN INTRINSIC B CELL DEFECT

	GENETICALLY DEFINED			UNDEFINED DEFECT OF CSR LOCATED IN RESPECT TO DNA CLEAVAGE	
CLINICAL MANIFESTATIONS	AID	UNG	PMS2	UPSTREAM	DOWNSTREAM
Patients (*n*)	55	3	4	16	15
Mean age at diagnosis in years	5	6	9	7	9
(range)	(0.3–53)	(3–9)	(5–20)	(1–15)	(0.3–23)
Recurrent infections (%)					
Upper respiratory tract	93	100	25	100	100
Lower respiratory tract	58	100	0	87	93
Digestive tract	17	66	0	31	20
Urinary tract	6	33	0	0	13
Central nervous system	14	0	0	0	0
Lymphoid hyperplasia (%)	75	66	0	50	40
Autoimmunity (%)	21	33	0	0	27
Cancers (%)	4	0	100	0	13

AID: Activation induced cytidinedeaminase, UNG: uracil-N glycosylase, PMS2: post meiotic segregation 2.

SHM on $CD19^+CD27^+$ B cells is dramatically reduced. Thus, AID deficiency leads to not only lack of CSR but also defective SHM (Revy et al., 2000) (Table 27.2).

Most of the patients exhibit enlarged secondary lymphoid organs, which may require surgical resection (tonsils) or biopsy (cervical lymph nodes); histological evaluation shows marked follicular hyperplasia. Germinal centers are giant, being 2 to more than 10 times larger than those from control reactive lymph nodes (Plate 27.I). The mantle zone and interfollicular areas appear thin. The giant germinal centers contain a normal follicular dendritic cell network and B cells that are PNA^+, $CD38^+$, $CD23^+$, $CD83^+$, $CD95^+$, $CD40^+$, IgM^+, $Bcl2^+$, and $Ki67^+$. Strikingly, many germinal-center B cells express sIgD, in contrast to normal reactive germinal centers, in which IgD^+ B cells are rarely found. The high proliferation index of germinal-center B cells is associated with a dense network of macrophages filled with apoptotic bodies, giving a starry-sky appearance. The characteristic markers and size of the germinal-center B cells identify them as proliferating ($Ki67^+$) germinal-center founder cells ($CD38^+$, $sIgM^+$, $sIgD^+$), prone to undergo SHM and selection (Lebecque et al., 1997). Occasional $CD27^+$ B cells as well as IgM- and IgD-expressing plasma cells are found in germinal centers and T-cell areas; however, neither IgG- nor IgA-expressing plasma cells are observed.

MOLECULAR BASIS OF AID DEFICIENCY

Because the analysis of informative pedigrees suggested an autosomal recessive inheritance, genetic mapping was attempted by studying the segregation of polymorphic microsatellite markers in several consanguineous families. Random screening of the genome indicated that disease segregation was compatible in all studied families with the telomeric region of the short arm of chromosome 12 at p13 with a multipoint LOD score of 10.45 (Revy et al., 2000). Recombination analysis defined the critical genetic interval as a 4.5 cM region, in which a gene coding for AID had recently been localized (Muto et al., 2000). The human *AICDA* gene encompasses about 10 kb of genomic DNA and is organized in five exons and four introns. Definition of the exon–intron boundaries allowed a search for mutations at the genomic level. Deleterious mutations were found in all regions of the gene, including the cytidine deaminase domain (Lee et al., 2005; Minegishi et al., 2000; Quartier et al., 2004; Revy et al., 2000).

Forty different mutations have been found in 45 families, most often as homozygous defects (29 families) and less frequently as compound heterozygous mutations (16 families). They include missense mutations (28 families), nonsense mutations, and small deletions (6 families) (Fig. 27.1). In addition, deletions of the entire coding region (3 families) or splice-site mutations (3 families) leading to either a longer RNA transcript or to frameshift and premature stop codon were reported. The same mutations were found in a number of unrelated families of the same ethnic origin (e.g., French Canadians, Turkish); analysis of flanking polymorphic markers in these families indicated a common ancestral origin of the mutation (Minegishi et al., 2000; Revy et al., 2000).

Several mutations, located in the C-terminal part of the *AICDA* gene, were recently shown to result in defective CSR, whereas SHM is not affected (Ta et al., 2003). This observation suggests that, in addition to its cytidine deaminase

Table 27.2 LABORATORY FINDINGS OF CSR-DS CAUSED BY AN INTRINSIC B-CELL DEFECTS

		GENETICALLY DEFINED (%)			UNDEFINED DEFECT OF CSR LOCATED IN RESPECT TO DNA CLEAVAGE (%)	
ASSAY	PHENOTYPE	AID	UNG	PMS2	UPSTREAM	DOWNSTREAM
Patients(*n*)		55	3	4	16	15
Ig Levels						
IgM	Normal	5	0	75	25	20
IgM	Elevated	95	100	25	75	80
IgG	<2 SD below normal	15	66	25	25	30
IgG	Undetectable	85	33	0	75	70
IgA	<2 SD below normal	17	66	50	30	30
IgA	Undetectable	83	33	0	70	70
Antibody Production						
Allohemagglutinins	Present	100	ND	ND	100	100
Polysaccharide IgM antibody	Present	100	ND	ND	100	100
IgG antibody production	Undetectable	100	100	ND	100	100
B-Cell Count and Function						
B-cell count	Normal	100	100	100	100	100
$CD27^+$ B-cell count	Normal	100	100	75	95	0
	Decreased	0	0	25	5	100
sCD40L-induced proliferation	Normal	100	100	100	100	100
sCD40L-inducedCSR	Undetectable	100	100	100	100	100
SHM	Normal	10	100 (biased)	100	100	100*
	Decreased	90	0	0	0	0

AID: Activation-induced cytidine deaminase, UNG: uracil-N glycosylase, PMS2: post meiotic segregation 2, CSR: class switch recombination, SHM: somatic hypermutation, sCD40L: soluble CD40 ligand

* normal SHM frequency on a decreased CD27+B cell subset

activity, AID acts on CSR by binding a CSR-specific cofactor. Because mutations and DNA double-strand breaks (Doi et al., 2009) are normally found in Sμ regions, a defect in targeting AID to the S regions is unlikely and a defect in DNA repair can be suspected (Barreto et al., 2003). Another unexpected finding recently reported (Imai et al., 2005; Kasahara et al., 2003) is that a heterozygous nonsense mutation, also located in the C-terminal domain, results in the loss of the nine last amino acids of NES ($AID^{\Delta C}$) and a variable Ig CSR deficiency condition transmitted as an autosomal dominant disease (Fig. 27.1). Haploinsufficiency, although reported in mice with weak consequences on Ig levels (Takizawa et al., 2008), is highly unlikely because all other human subjects heterozygous for AID deficiency always exhibit normal Ig levels. The accumulation of AID devoid of normal NES in the nucleus overriding the wild-type AID (Ito et al., 2004) could account for this phenotype, an hypothesis reinforced by the in vitro study of artificial mutants (Doi et al., 2009; Patenaude et al., 2009).

URACIL-N-GLYCOSYLASE DEFICIENCY

URACIL-N-GLYCOSYLASE

Uracil-N-glycosylase (UNG) belongs to the family of uracil-DNA-glycosylases capable of deglycosylating uracil residues that are misintegrated into DNA. Each of the two different UNG promoters creates a different isoform: UNG1 (305 amino acids), which is mitochondrial and ubiquitinously expressed, and UNG2 (314 amino acids), which is nuclear and expressed in proliferating cells, including B cells undergoing CSR. The catalytic domain binds the replication protein A (RPA) and the proliferating cell nuclear antigen (PCNA) in a multimolecular complex involved in DNA base excision repair (Fig. 27.2). According to a model proposed by Petersen-Mahrt and Neuberger (2002), AID deaminates cytosine into uracil residues on single-stranded DNA. Following the deglycosylation and removal of uracil residues by UNG, an abasic site is created that can be attacked by an apyrimidinic endonuclease

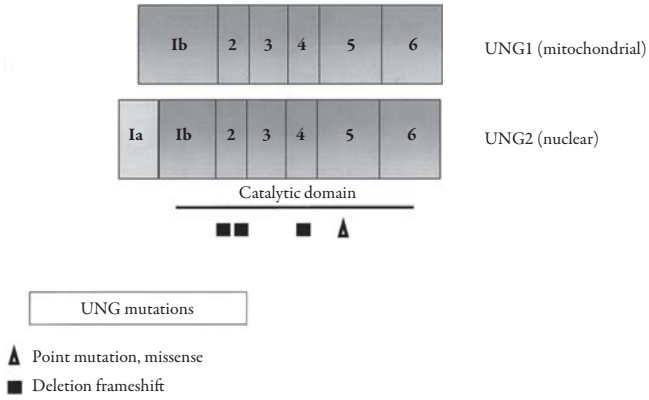

Figure 27.2 Schematic representation of uracil-N-glycosylase (UNG) and localization of mutations in UNG deficiency. Mutations affect the catalytic domain of both UNG 1 (mitochondrial isoform) and UNG2 (nuclear isoform).

(APE), leading to single-stranded DNA breaks. The processing and repair of the DNA nicks complete both CSR and SHM. In the absence of UNG, this pathway is impaired, resulting in defective CSR and abnormal SHM. The presence of SHM with a skewed pattern of G/C residues may arise from the replication of U/G lesions in the absence of U removal. MMR enzymes may also recognize and repair these lesions, introducing mutations on neighboring nucleotides that result in transitions and transversions of A/T residues (Petersen-Mahrt et al., 2002; Rada et al., 2002). Alternatively it has been proposed that rather than UNG's enzymatic activity on DNA, the UNG protein is required for CSR by its involvement in the recruitment of DNA repair molecules or its role in folding the CSR-induced DSBs (Honjo et al., 2004). However, this hypothesis does not explain the defect in DSB formation within Sμ regions observed in UNG-deficient B cells (Imai et al., 2003b).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF UNG DEFICIENCY

To date, only three UNG-deficient patients (MIM #608106, gene * 191525) have been reported (Imai et al., 2003b); two were diagnosed during childhood and the other was diagnosed as an adult. All three patients have had a history of frequent bacterial infections of the respiratory tract that are easily controlled by regular IVIG infusions. Lymphadenopathy was observed in two of the three patients, one with impressive enlargement of the mediastinal lymph nodes. The adult patient has developed Sjögren syndrome in recent years. The absence of metabolic abnormality suggests that UNG deficiency is compensated in mitochondria by other uracil-DNA-glycosylases (Table 27.1).

LABORATORY FINDINGS IN UNG DEFICIENCY

At the time of diagnosis, all three patients presented with Ig CSR deficiency, defined by markedly diminished serum levels of IgG and IgA and an increased serum level of IgM. No antibodies of the IgG isotype were detected to vaccines nor to infectious agents.

Peripheral blood T-cell and T-cell–subset numbers as well as in vitro T-cell proliferation to mitogens and antigens were normal. Peripheral blood B cells ($CD19^+$) were normal in number; expressed CD40, which was of wild type; and co-expressed sIgM and sIgD with a normal fraction expressing CD27.

When cultured in the presence of sCD40-L, B cells proliferate normally but fail to undergo CSR and secrete IgG, IgA, or IgE in the presence of appropriate cytokines. As in AID deficiency, the CSR defect occurs downstream from S-region transcription and upstream from DSBs in Sμ regions.

Somatic hypermutations were found in normal frequency in $CD19^+CD27^+$ B cells but exhibited a biased pattern. Almost all mutations are transitions at G/C residues (G > A, C > T), although transitions and transversions are equally present on A/T nucleotides (Table 27.2).

The association of an Ig CSR deficiency phenotype with UNG deficiency, together with the description of a mild CSR defect and a similarly biased SHM in UNG-deficient mice, provides a strong argument for DNA-editing activity of AID (Di Noia and Neuberger, 2002; Rada et al., 2002).

MOLECULAR BASIS OF UNG DEFICIENCY

Four different mutations affecting the catalytic domain of UNG1 and UNG2 have been found. Two patients have small deletions leading to a premature stop codon (homozygous

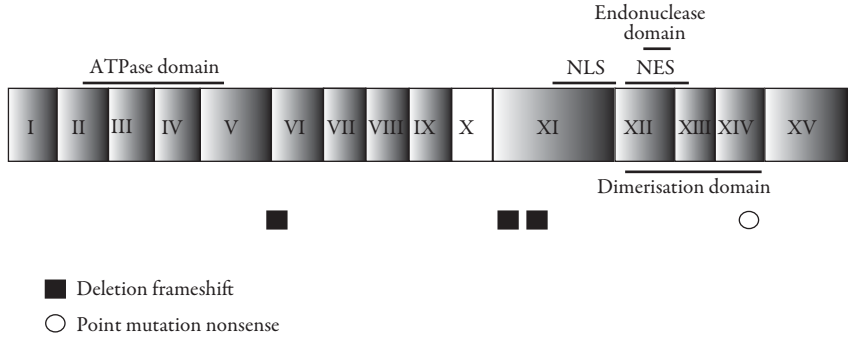

Figure 27.3 Schematic representation of PMS2 and localization of mutations in Ig CSR deficiencies associated with PMS2 defect. All mutations lead to predicted truncated proteins.

mutation in one patient born in a consanguineous family and two heterozygous mutations in the other). The third patient carries a homozygous missense mutation (Fig. 27.3). UNG expression and function were defective in Epstein-Barr virus (EBV) B-cell lines, providing evidence for the lack of any compensatory uracil-DNA glycosylase activity, at least in B cells.

POSTMEIOTIC SEGREGATION 2 DEFICIENCY

POSTMEIOTIC SEGREGATION 2

Postmeiotic segregation 2 (PMS2) belongs to the MMR pathway that recognizes and repairs mismatched nucleotides on DNA. The MMR system is known to play a role in CSR in mice, as shown by abnormal switched isotype levels and switch junctions (Ehrenstein et al., 2001; Schrader et al., 2002). There are two main MMR components: the MutS homolog (MSH1–6) and the MutL homolog (PMS2/MLH1/PMS1). The MSH2–MSH6 complex appears to recognize AID-induced DNA mismatches in the absence of UNG, leading to backup CSR and SHM, as shown by the phenotype of a double UNG-MSH2 knockout mutant (Rada et al., 2004). Recently, it has also been reported that MSH5 variants in humans can be associated with common variable immunodeficiency (CVID) and IgA deficiency phenotypes, including abnormal switch junctions that are characteristic of DNA repair defects (Sekine et al., 2007), although these results are controversial (Guikema et al., 2008). The role of the PMS2–MLH1 complex is less clear. Recently, it has been proposed that the MMR system can convert DNA SSBs into DSBs in Sμ regions upon CSR activation (Stavnezer and Schrader, 2006). In the SHM process, the repair of V regions requires the MMR and error-prone DNA polymerases. The MSH2–MSH6 complex is essential in SHM for recognizing the AID-induced U/G mismatch, and recruiting exonuclease (EXO1) and polymerase eta (Delbos et al., 2007). The role of the PMS2–MLH1 complex in SHM remains subject to debate (Kim et al., 1999; Phung et al., 1999).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF PMS2 DEFICIENCY

Heterozygous PMS2 mutations are associated with susceptibility to colorectal carcinoma (Hendriks et al., 2006), whereas homozygous mutations are responsible for early occurrence of various cancers (colorectal adenocarcinoma, T lymphoma, T acute lymphoid leukemia; Wimmer and Etzler, 2008). Another feature of the disease is the presence of café-au-lait skin spots. One patient (7 years of age) was referred to our unit for peculiar susceptibility to bacterial infections 2 years before developing colonic adenocarcinoma. The three other patients had no remarkable history of repeated infections before developing cancers.

LABORATORY FINDINGS

IMMUNOGLOBULIN LEVELS AND ANTIBODY PRODUCTION

The first patient was diagnosed as suffering from a CSR deficiency since serum IgG levels were markedly diminished and IgA was undetectable. She therefore received regular IVIG substitution. The three other patients presented with undetectable serum IgG2/IgG4 isotypes, associated with transient IgA decrease in one (Table 27.2).

T and B Lymphocytes

Although tested several years after chemotherapy, all four patients presented with a mild lymphopenia (around 1,000/mL). Peripheral blood T-cell ($CD3^+$) and T-cell–subset numbers ($CD4^+$ and $CD8^+$) as well as in vitro T-cell proliferation to mitogens and antigens were normal.

Peripheral blood B-cell ($CD19^+$) counts were normal. All $CD19^+$ B cells co-expressed sIgM and sIgD and a reduced fraction expressed CD27.

In vitro sCD40-L–induced B-cell proliferation was found to be slightly decreased compared to controls; B lymphocytes weakly undergo CSR toward IgA or IgE under activation by sCD40L and appropriate cytokines. As in AID or UNG deficiency the CSR defect occurs downstream from S-region transcription and upstream from the DSBs in Sμ regions.

SHM frequency was found to be slightly decreased in $CD19^+CD27^+$ B cells, but the SHM pattern was normal.

MOLECULAR BASIS OF PMS2 DEFICIENCY

The four patients carry homozygous nonsense mutations in the *PMS2* gene, leading to either a truncated protein or a lack of expression (Fig. 27.3). Expression of MLH1, PMS2's partner in MutLa, was slightly reduced but still present in nuclei, likely because of dimerization with PMS1. The decreased occurrence of DSBs in Sμ regions upon CSR activation in PMS2-deficient human B cells strongly suggests a role for PMS2 in DNA cleavage, likely through its endonucleasic domain. Since UNG-deficient patients exhibit a complete CSR defect, it appears that PMS2 does not play a role in CSR as an alternative pathway, but interacts downstream from UNG in the same pathway (Peron et al., 2008).

Ig CSR DEFICIENCIES WITH UNKNOWN MOLECULAR DEFECT(S)

Not all cases of Ig CSR deficiency due to an intrinsic B-cell defect are related to AID or UNG deficiency. Although most of these cases are sporadic, the mode of inheritance observed in a few multiplex or consanguineous families is compatible with an autosomal recessive pattern. The clinical phenotype is

similar to that of AID deficiency, including increased susceptibility to bacterial infections of the respiratory and gastrointestinal tracts. Lymphoid hyperplasia is milder and less frequent (50 percent), consisting of moderate follicular hyperplasia without the giant germinal centers typical of AID deficiency (Table 27.1).

The CSR defect appears to be milder, as residual serum levels of IgG can be detected in some patients. While isohemagglutinins and IgM antipolysaccharide antibodies are present in normal amounts, IgG antibodies against immunization antigens or infectious agents cannot be detected. Upon activation with sCD40L and cytokines, B cells proliferate but do not undergo CSR (Table 27.2).

The definition of the precise location of the CSR defect has led to the delineation of two distinct groups.

CSR DEFECT LOCATED UPSTREAM FROM S-REGION DNA CLEAVAGE

We are following a subgroup of 16 patients with an Ig CSR deficiency phenotype characterized by good prognosis, lack of autoimmune manifestations, and no increased risk of lymphoma or other malignancies. No CSR-induced DSBs occur in Sμ regions of patients' B cells, although both AID and UNG transcripts are normally expressed. The defect, located downstream from the S-region transcription and upstream from the S-region DNA cleavage, is restricted to CSR, as SHM is normal in both frequency and pattern in the $CD27^+$ B-cell subset, which is represented in normal numbers.

This type of Ig CSR deficiency could thus be caused by a direct or indirect impairment of AID targeting on S regions. Although targeting factors of AID are at present unknown, they most likely exist because AID deaminates cytosines only in the S and V regions in B cells. AID shares sequence similarity with the RNA-editing enzyme APOBEC-1, which is expressed only in the gut and requires a cofactor, ACF (APOBEC-1-cofactor), which targets APOBEC-1 on a unique C residue in ApoB mRNA. In addition, specific switch factors have been described in CSR-activated B cells. Although their role remains elusive, it has been proposed that these cofactors act as docking proteins for the recruitment of the recombinase complexes to DNA-specific regions (Ma et al., 2002; Shanmugam et al., 2000).

CSR DEFECT LOCATED DOWNSTREAM FROM S-REGION DNA CLEAVAGE

This condition has been found in 15 patients thus far (Imai et al., 2003a). The prognosis of this Ig CSR deficiency subgroup is complicated by the occurrence of autoimmune manifestations that were found in 4 of 15 patients and were sometimes life-threatening (severe autoimmune hemolytic anemia). DSBs are normally detected in Sμ regions in CSR-activated B cells, suggesting a defect downstream from DNA cleavage. The subsequent step, DNA repair, is impaired because excision circles and functional transcripts of switched isotypes cannot be detected. The normal presence of DSBs and of mutations in Sμ regions rules out a defect in AID targeting to S regions. SHM is present in normal frequency and pattern in the purified $CD27^+$ B-cell population. However, the $CD27^+$ B-cell count is decreased compared to that of controls (<10 percent of the B-cell population).

Two hypotheses can account for this unique phenotype: (1) a defect in survival signals delivered to switched B cells (this does not fit with the observed defective in vitro CSR) and (2) a DNA repair defect, because CSR-induced DSBs occur normally. CSR and SHM are known to use different pathways for DNA repair. NHEJ enzymes and the MRE11/hRad50/NBS1 protein complex have been shown to be involved in CSR DNA repair (Casellas et al., 1998; Rolink et al., 1996). Nevertheless, a defect in one of these proteins is unlikely, given the phenotype of patients carrying mutations in *MRE11* or *NBS1* genes (ataxia-like disease or Nijmegen breakage syndrome, respectively). A defective CSR with normal SHM has been reported in H2AX and 53BP1 knockout mice (Manis et al., 2004; Petersen et al., 2001; Ward et al., 2004), but a defect in one of these genes has been ruled out in our patients by sequence analysis. Thus, other DNA repair factor(s) have to be considered, including the undefined cofactor that binds to the C-terminal part of AID. Two of our 15 patients have developed Hodgkin's lymphoma. Although the number is small, this observation is compatible with a DNA repair defect, which could facilitate illegitimate recombination leading to oncogene activation.

STRATEGY FOR DIAGNOSIS OF HYPER-IGM SYNDROME DUE TO AN INTRINSIC B-CELL DEFECT

Like all patients with the diagnosis of Ig CSR deficiency, this group of patients with an intrinsic B-cell defect is defined by markedly diminished serum levels of IgG and IgA and a normal or increased level of IgM. Assessment of $CD27^+$ B-cell count and analysis of the frequency and pattern of SHM allow identification of these different autosomal recessive Ig CSR deficiency conditions (Table 27.2). A precise diagnosis is required for optimal prognosis assessment.

Other Ig CSR deficiency syndromes, not caused by a B-cell–specific abnormality, are associated with T-cell defects, which strongly worsens the prognosis. Defects of CD40L and CD40 are excluded by assessing membrane expression of these two molecules and/or direct gene sequencing. A defect in the NF-κB activation pathway (due to mutations in *NEMO*, also designated IKK-γ, or in IκBα) is often responsible for an Ig CSR deficiency phenotype characterized by a T-cell defect and frequently associated with an hidrotic ectodermal dysplasia.

Other causes of Ig CSR deficiency that should be excluded are the following:

1. Ataxia-telangiectasia (AT), in which elevated IgM can develop before the onset of neurological manifestations (Gatti et al.,1991; Meyts et al., 2003). The CSR defect is related to an intrinsic B-cell defect since AT-mutated (*ATM*), the gene responsible for this syndrome, plays

a role in CSR (but not SHM) DNA repair (Pan-Hammarstrom et al., 2003).

2. Congenital rubella, in which a defect of T-cell activation leads to defective CD40L expression on $CD4^+$ T cells (Kawamura et al., 2000).
3. Patients with major histocompatibility complex (MHC) class II deficiency have diminished expression of CD40L by activated $CD4^+$ T cells and can present with elevated serum IgM (Nonoyama et al., 1998).
4. An Ig CSR deficiency phenotype has been observed as part of combined immunodeficiencies linked to leaky mutations in genes encoding factors for NHEJ, such as Cernunnos, DNA ligase IV, or RAG (Buck et al., 2006).

PRENATAL DIAGNOSIS

Prenatal diagnosis can be performed by sequence analysis of the *AID* or *UNG* gene with genomic DNA obtained from the fetus. Prenatal diagnosis could raise ethical problems since the prognosis is generally good and nearly all patients reach adulthood with only a few recurrent bacterial infections, provided that prophylactic treatment with IVIG is started early and given on a regular basis. However, other life-threatening complications (autoimmunity, lymphoma) can occur, and UNG-deficient patients, as shown by the ung-deficient mice, and especially PMS2-deficient patients are prone to tumors.

PROGNOSIS AND TREATMENT

As soon as the diagnosis of Ig CSR deficiency is established, treatment with regular IVIG infusions (400–600 mg/kg every 21–28 days) must be initiated with the aim of maintaining a trough IgG level of 700 to 800 mg/dL. This protocol results in a significant reduction in the severity and frequency of infections and often reduces or even normalizes serum IgM levels. However, the lymphoid hyperplasia does not seem to be reduced by IVIG therapy.

With adequate prophylactic treatment, patients with Ig CSR deficiency due to intrinsic B-cell deficiency are protected from infections and can reach adulthood without developing bronchiectasis. Surprisingly, they do not appear to be unusually susceptible to enteroviral infections, a serious complication observed in patients with X-linked agammaglobulinemia (Quartier et al., 2000). This observation suggests a protective role of IgM (even without SHM) as a first barrier against some pathogens.

Autoimmunity has been reported in AID deficiency and in the Ig CSR deficiency condition characterized by a CSR defect located downstream from the S-region cleavage. Both conditions are characterized by an SHM defect (in the latter condition the B-cell memory subset is decreased). In contrast, autoimmunity has not been reported in the Ig CSR deficiency condition characterized by a CSR defect located upstream from the S-region cleavage, in which both SHM and memory B-cell numbers are normal. Autoimmunity could thus be related to defective SHM. It may be that autoimmunity, initiated by germline Ig sequences, is caused by the lack of negative selection in germinal centers.

A more serious complication, the occurrence of tumors, has to be considered for the prognostic assessment. The subgroup of Ig CSR deficiency characterized by a CSR defect located downstream from the S-region cleavage may be directly related to a DNA repair defect and patients could become susceptible to an increased risk of malignancy. UNG is part of the DNA base excision repair and is thus involved in the repair of spontaneously occurring base lesions as part of a major antimutagenic defense strategy. Interestingly, UNG-deficient mice develop B-cell lymphomas when aging (Nilsen et al., 2003), so UNG deficiency may predispose to malignancies in adulthood. Moreover, the hallmark of PMS2 deficiency is the occurrence of tumors from the first years of age (Wimmer and Etzler, 2008).

ANIMAL MODELS OF AID, UNG, AND PMS2 DEFICIENCIES

No naturally occurring mutants defective in AID, UNG, or PMS2 have been described in animals. However, AID- and UNG- and PMS2-deficient mice, as well as AID-transgenic mice, have been generated by gene-targeting techniques.

AID-DEFICIENT MICE

The phenotype of $AID^{-/-}$ mice resembles completely that observed in patients and is associated with the three main features of AID deficiency:

1. Defective CSR in vivo and in vitro. The CSR defect has been shown to occur downstream from S-region transcription and upstream from the occurrence of repair foci at DSBs on the Ig locus (Petersen et al., 2001).
2. Defective somatic hypermutation.
3. Giant germinal-center formation in spleen and lymph nodes with accumulation of $IgM^+/IgD^+/PNA^+$ proliferating B cells (Muramatsu et al., 2000).

AID-TRANSGENIC MICE

AID-transgenic mice express mutations in the T-cell receptor, a finding indicating that aberrantly expressed and overexpressed AID can exert its activity outside the B-cell compartment. All of these mice develop tumors affecting the lymphoid compartment (T lymphoma) and nonlymphoid tissues (e.g., epithelium of respiratory bronchioles). Interestingly, these transgenic mice do not develop B-cell lymphomas, an observation suggesting that AID activity and/or nuclear localization can be regulated effectively only in B cells (Okazaki et al., 2003).

UNG-DEFICIENT MICE

UNG-deficient mice have been generated to study the effects of base excision repair defects on the incidence of tumors (Nilsen et al., 2000). No striking biological effects could be shown, most likely because of compensatory mechanisms provided by other uracil-DNA glycosylases. A more recent study of CSR and SHM provided evidence for a mild in vivo and a profound in vitro CSR defect. The remaining CSR observed in vivo is likely related to MMR enzymes, which are known to be involved in CSR in mice (Rada et al., 2002). This study also showed a normal frequency of SHM that exhibit a skewed pattern. Since all mutations at G/C residues are transitions, likely occurring on U/G nucleotides after replication, mutations observed on A/T residues could be the consequence of the recognition and repair of U/G lesions by MMR enzymes. Both observations, confirmed by similar data in humans (although the CSR defect is much more pronounced in UNG-deficient patients), are a strong argument for a DNA-editing activity of AID.

Without exception, UNG-deficient mice develop B lymphomas when aging, an observation that could reflect the lack of compensatory uracil-DNA-glycosylases in B cells (Nilsen et al., 2003). Another adverse consequence has been reported in UNG-deficient mice: compared with wild-type mice, postischemic brain injury is much more severe, a complication likely related to the mitochondrial DNA repair defect (Endres et al., 2004).

PMS2-DEFICIENT MICE

B-cell–mediated immunity has been studied for a long time in MMR-deficient mice. The phenotype is different according to the inactivated enzyme: MSH2- or MSH6-deficient mice exhibit mild CSR defect with normal switch junctions but a skewed pattern of SHM (Martomo et al., 2004; Schrader et al., 2002). In contrast, PMS2- or MLH1-deficient mice exhibit a defective CSR with abnormal switch junctions but normal SHM (Ehrenstein et al., 2001; Kim et al., 1999; Schrader et al., 2002). It is however likely that the MMR enzymes do not play an equivalent function in CSR in humans and in mice since MSH2/MSH6 can compensate UNG deficiency in mice but not in humans.

CONCLUSIONS

The ongoing investigation of inherited Ig CSR deficiencies is shedding new light on the process of antibody maturation in human B cells. This is especially true for the evaluation of Ig CSR deficiencies due to an intrinsic B-cell defect, which has illustrated the complex mechanisms involved in both events of B-cell maturation—CSR and SHM. On the basis of clinical phenotypes, the following mode of action of AID and UNG is proposed: AID, at center stage of CSR and SHM, deaminates cytosine nucleotides to uracil in S and V regions of Ig genes. The integrated uridine nucleosides are deglycosylated and removed from DNA by UNG, resulting in an abasic site that becomes a target for an endonuclease. This DNA cleavage step appears to be sufficient for SHM, at least on G/C residues, but not for CSR, which requires DSB. Beyond its cytidine deaminase activity, AID requires specific cofactors. A CSR-specific cofactor, which could be involved in DSB DNA repair, is expected to bind to the C-terminal part of multimeric AID. It is likely that the molecular definition of the Ig CSR deficiency caused by a CSR defect located downstream from S-region cleavage will lead to the identification of this cofactor. Taken together, these observations indicate that AID acts in a multimolecular complex composed of individual partners that have yet to be defined. The precise diagnosis of these Ig CSR deficiencies, in addition to contributing to a better understanding of the antibody maturation processes, provides science-based guidelines for better prognosis assessment and treatment management.

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28.

GENETIC APPROACH TO COMMON VARIABLE IMMUNODEFICIENCY AND IGA DEFICIENCY

Lennart Hammarström

Common variable immunodeficiency (CVID) affects approximately 1 in 10,000 to 100,000 individuals and is equally distributed between the sexes. It is likely that it consists of a number of genetically distinct disorders with a common phenotype. The patients display a marked reduction in serum levels of both IgG and IgA, and IgM levels are reduced in half of the patients. The age of onset of disease was previously suggested to show two peaks (Hermaszewski and Webster, 1993) but on reevaluation it seems to be a continuous process (Chapel et al., 2008). The disorder may appear in previously immunologically normal individuals, although the induction phase has been documented only in a few cases. Usually, the patients present with clinical symptoms due to the hypogammaglobulinemia, as they suffer from frequent respiratory and gastrointestinal tract infections (Table 28.1). Mortality is high and was recently estimated to be 19.6 percent, a significantly shorter survival than age- and sex-matched controls (Resnick et al., 2012). In addition to the B-cell defect, variable degrees of T-cell dysfunction have frequently been noted in CVID patients. Usually, low or normal proportions of $CD19^+$ cells are observed, a finding that distinguishes "classical" CVID patients from those with a concomitant thymoma.

Selective IgA deficiency (IgAD), based on 0.07 g/L of serum IgA as the upper limit for diagnosis, is the most common form of immunodeficiency in the Western world and affects approximately 1 in 600 individuals. Marked variability in the prevalence can, however, be found in different ethnic groups. A lower frequency has been reported in certain Asian populations, suggesting a genetic influence. In about two thirds of the cases, the deficiency does not lead to an increased occurrence of infections, whereas the remaining patients suffer from infections in both the upper and lower respiratory tract (Table 28.1). The defect is manifested already at the stem-cell level, and transfer of bone marrow from an IgA-deficient donor to a normal fully conditioned recipient results in IgA deficiency in the recipient (Hammarstrom et al., 1985a), whereas transfer of bone marrow from a normal individual to an IgA-deficient patient will correct the defect (Kurobane et al., 1991). The silent genes can be reexpressed in the children of IgA-deficient parents (Hammarstrom et al., 1987), strongly suggesting that the defect is due to dysregulation of the expression of the immunoglobulin (Ig) genes. In a few selected cases, the defect is restricted to one of the two IgA subclasses, and these cases are most often due to deletions of the corresponding α chain gene (*IGHA*) (Lefranc et al., 1991; Migone et al. 1984).

By definition, IgAD is expected to be selective and confined to the IgA class. However, in some cases, a simultaneous change in the IgG subclass pattern is seen with a lack of specific antipolysaccharide antibodies of the IgG2 subclass (Hammarstrom et al., 1985b) or a total lack of serum IgG2 (Oxelius et al., 1981), IgG4, and IgE (Hammarstrom et al., 1986a), reflecting a relative or absolute block in switching to genes downstream of α1. Progression from CVID has been observed in a few cases (for review see Aghamohammadi et al., 2008), suggesting that the two diseases are in fact related and may represent facets of the same underlying defect. However, the overlap appears to be smaller than previously suggested.

During the past few years, mutations in a number of genes associated with B-cell differentiation, including *ICOS*, TACI (*TNFRSF13B*), *CD19*, *MSH5*, BAFF receptor (BAFFR) *TNFRSF13C*, *CD21*, and *CD81* (for references, see below), have been found in patients with CVID. However, the molecular basis still remains unknown in the vast majority of cases.

HISTORICAL OVERVIEW

The first report of Ig deficiency appeared in 1952 (Bruton) and inspired a large number of investigators to analyze Ig

Table 28.1 CLINICAL VARIABLES IN IMMUNOGLOBULIN-DEFICIENT PATIENTS

SYMPTOM	CVID	IGAD	COMMON PATHOGENS
Sinusitis	+++	++	*Haemophilus influenzae*
	+++	+	*Streptococcus pneumoniae*
	+++	+	*Moraxella catarrhalis*
Pneumonia	+++	(+)	*Haemophilus influenzae*
	+++		*Streptococcus pneumoniae*
Bronchiectasis	++		
Gastrointestinal infections	+	(+)	*Giardia lamblia*
	+		*Campylobacter jejuni*
Splenomegaly	+		
Lymphadenopathy	++		
Conjunctivitis	+		
Meningitis	+		ECHO virus
Viral infections	+		Hepatitis C, varicella-zoster virus
Cancer risk	+		
Autoimmunity	+	+	
Hemolytic anemia	(+)		
Thrombocytopenia	(+)		
Urogenital infection	(+)		*Ureaplasma urealyticum*

+++ = very frequent

++ = frequent

+ = occasional

(+) = infrequent

levels in patients prone to infections (reviewed in Good et al., 1962; Smith and Notarangelo, 1997). At the time, knowledge on lymphocyte subsets was lacking, and patients with autosomal recessive severe combined immunodeficiency were sometimes referred to as having agammaglobulinemia (Keidan et al., 1953). Females with CVID were already reported the year after Bruton's original description (Olhagen, 1953). Subsequently, both adult-onset (Grant and Wallace, 1954; Jacobsen, 1954; Lang et al., 1954; Olhagen, 1953; Prasad and Koza, 1954; Saslaw and Wall, 1954) and congenital agammaglobulinemia in females (Pearce and Perinpanayagam, 1957) were described. Adult onset in male patients with a CVID phenotype was also reported (Olhagen, 1953), as reviewed in Citron (1957), who also described additional patients suffering from agammaglobulinemia with concomitant splenomegaly.

IgAD was first described in 1964, being noted in two healthy laboratory staff, clearly suggesting that IgA may be dispensable in certain individuals (Rockey et al., 1964). However, based on a review of 30 cases, it was subsequently determined that this defect frequently results in a propensity for infections (Ammann and Hong, 1971).

The classification of Ig deficiencies, including CVID and IgAD, is regularly being updated by the IUIS Expert Committee on Primary Immunodeficiencies, with the most recent report having been published in 2011 (al-Herz et al., 2011).

MODE OF INHERITANCE

Familial inheritance of the deficiency is observed in approximately 20 percent of the cases, and CVID and IgAD may both be present in the same family. In rare instances, patients with IgAD may progress to CVID, suggesting that the disorders may reflect facets of the same underlying disease in a limited subgroup of patients (for review see Aghamohammadi et al., 2008). The finding of Ig deficiency in only one of a set of monozygotic twins (Alper et al., 2006; Huntley and Stephenson, 1968, Lewkonia et al., 1976, Ulfarsson et al., 1982) suggests an environmental, possibly infectious, agent as a triggering factor.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

INFECTIONS

Patients with IgAD and CVID suffer from a variety of respiratory and gastrointestinal tract infections (Table 28.1). The severity of infections reflects the degree of immune dysfunction and is thus more pronounced in patients with profound hypogammaglobulinemia. Enlarged lymph nodes and spleen are noted in up to half of the patients with CVID, but not in patients with X-linked agammaglobulinemia (XLA) (Curtin et al., 1995), and may reflect persistence of low-grade infections. The lymph nodes may show a striking reactive follicular hyperplasia, and noncaseating granulomas may be present in the liver (Fig. 28.1) and skin (Fig. 28.2). The gastrointestinal tract may also be involved in this process, with a characteristic nodular lymphoid hyperplasia. The spectrum of infections in IgAD and CVID patients is, however, usually rather limited, and some of these are highlighted below.

Haemophilus influenzae is a common pathogen in the human respiratory tract. The majority of isolates are nonencapsulated and fall into the category of serologically nontypable strains, which often cause acute otitis media, acute sinusitis, conjunctivitis, pneumonia, and bronchitis in immunocompetent patients. These bacteria also constitute the major pathogen in CVID patients who suffer from frequent upper respiratory tract infections. The patients may remain colonized for years with the same strain in spite of "adequate" gammaglobulin therapy and antibiotics (Samuelson et al., 1995) and may develop multiple bouts of symptoms. It is still unclear why the bacteria are poorly cleared by antimicrobial therapy, but intracellular growth may occur in crypt cells in the adenoids and tonsils.

Moraxella catarrhalis and *Streptococcus pneumoniae* are the second and third most common pathogens in patients with CVID; both organisms give rise to upper respiratory

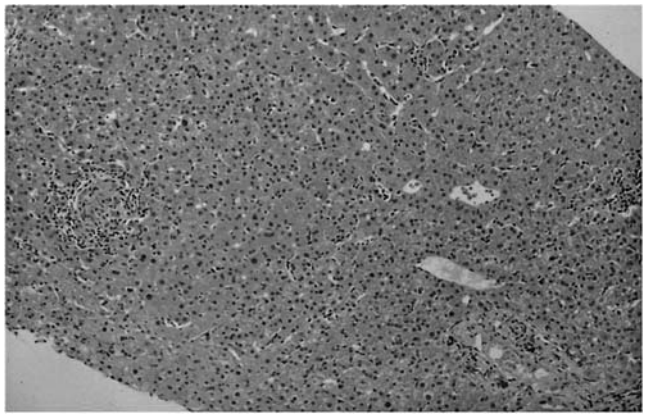

Figure 28.1 Granulomas in the liver of 47-year-old CVID patient. (See Color Plate.)

tract infections. In series of patients published earlier, serious pulmonary manifestations such as bronchiectasis, pulmonary fibrosis, and respiratory failure were not uncommon (Bjorkander et al., 1984; Dukes et al., 1978; Hausser et al., 1983). However, increasing awareness of immunodeficiency disorders, early diagnosis, and prompt institution of antibiotic therapy and gammaglobulin substitution have resulted in a far lower frequency of severe respiratory tract complications.

Mycoplasma/ureaplasma have been increasingly recognized as pathogens in humans, and hypogammaglobulinemic patients appear susceptible to these infections, but the diagnosis may be delayed due to the insidious onset of symptoms. CVID patients may suffer a protracted clinical course, and severe sequelae have been reported in a number of patients, including extensive joint destruction (Fig. 28.3).

Transient recovery of the ability to produce immunoglobulins has been reported in a few CVID patients at the time of hepatitis C infection (Hammarstrom and Smith, 1986b; Osur et al., 1987) and has also been demonstrated in patients infected with HIV (Morell et al., 1986; Webster et al., 1986; Wright et al., 1987), suggesting an immunoregulatory dysfunction as a cause of CVID in some patients.

INFLAMMATORY BOWEL DISEASE

A few IgAD patients and 6 to 10 percent of CVID patients have previously been described who suffer from Crohn's disease or ulcerative colitis, but an association has not yet been ascertained due to the lack of a sufficiently large number of patients.

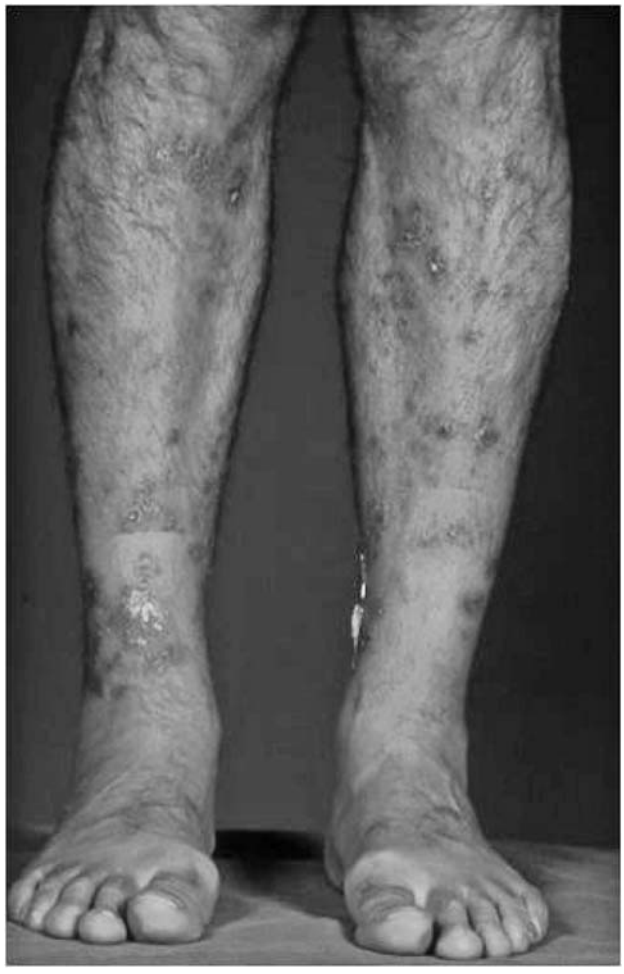

Figure 28.2 Cutaneous granulomas on the legs of a 47-year-old man with CVID. (See Color Plate.)

CANCER INCIDENCE

A prospective study from 1985 (Kinlen et al., 1985) reported a 23-fold increased risk for malignant lymphoma and a 50-fold increase for gastric cancer among 377 patients with hypogammaglobulinemia, primarily CVID. Patients were followed for an average of 10 years. In another study, 98 patients with CVID were followed for up to 13 years, and the risk for lymphomas was estimated to be more than 100-fold increased (Cunningham-Rundles et al., 1987). However, only a slightly increased incidence in malignant lymphoma and stomach cancer was noted in Swedish CVID patients followed for 20 years, and no increased risk for malignancy could be observed in a large cohort of IgAD patients (Mellemkjaer et al., 2002).

AUTOIMMUNITY

The list of autoimmune disorders associated with IgAD and CVID has grown to include, among others, systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren syndrome, dermatomyositis, thyroiditis (Graves disease), celiac disease, insulin-dependent diabetes mellitus, pernicious anemia, Addison disease, idiopathic thrombocytopenic purpura (ITP), and autoimmune hemolytic anemia. This is in stark contrast to X-linked agammaglobulinemia, where autoimmunity is rarely observed (see Chapter 25). In a very early survey of IgAD patients, 37 percent were in fact found to have autoimmune disease or autoimmune phenomena (Ammann and Hong, 1971). First-degree relatives of IgAD patients also show a higher-than-expected prevalence of autoantibodies (Jorgensen et al., 2009), suggesting a sharing of genetic factors between IgAD and susceptibility for autoimmunity;

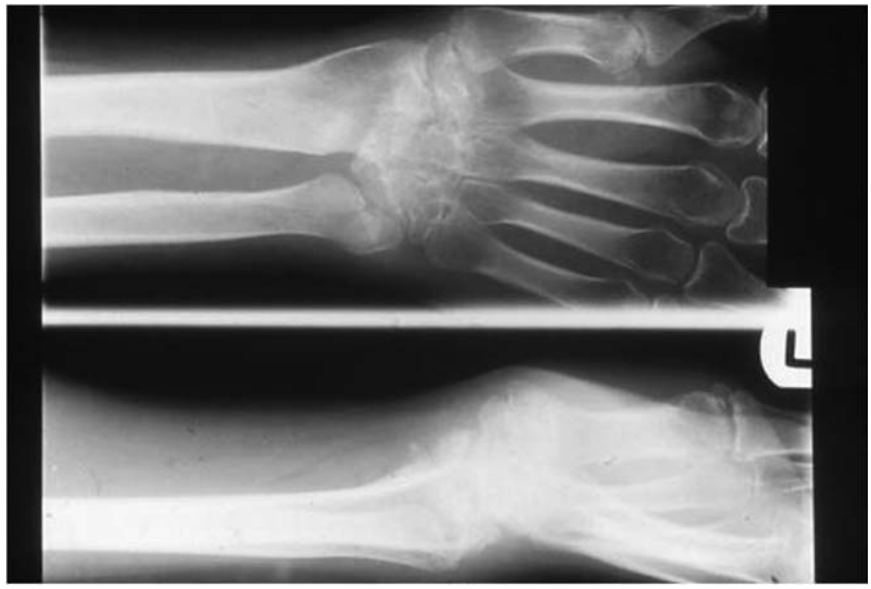

Figure 28.3 Destruction of carpal bones and subluxation of wrist joint in a 42-year-old patient with CVID and *Mycoplasma hominis* infection.

this notion was recently confirmed in a large cohort of IgAD patients (Ferreira et al., 2010).

ANTI-IGA ANTIBODIES

Antibodies against IgA are a common finding in patients with IgAD or CVID, and up to 40 percent show demonstrable titers. The etiology of this immune response is as yet unknown, as approximately 20 to 40 percent of the patients with IgAD have developed anti-IgA antibodies at the time of diagnosis without being exposed to IgA-containing blood products. Antibodies against IgA are usually of the IgG class, but IgM anti-IgA antibodies may also be present (Bjorkander et al., 1987). In exceptional cases, the antibodies are of the IgE class (Burks et al., 1986; Ferreira et al., 1988), and a few severe transfusion reactions have been attributed to these autoantibodies (Rachid et al., 2011).

Maternal transmission of IgAD, mediated by placentally transferred anti-IgA antibodies, could theoretically contribute to induction of the deficiency in selected cases (de Laat et al., 1991; Petty et al., 1985), and IgAD is overrepresented in children born to mothers with anti-IgA antibodies.

LABORATORY FINDINGS

The pathognomonic hallmark of IgAD is a low level of serum IgA (<0.07 g/L) with normal levels of IgM and IgG (Table 28.2). However, raised levels of IgG, mainly IgG1 and IgG3, may be seen in up to a third of the patients. Almost invariably, there is a concomitant lack of secretory IgA, although a dichotomy has been documented in exceptional cases.

In CVID, IgA is usually absent and serum IgG levels are decreased (≤3 g/L), whereas IgM is low or absent in only half of the cases (Table 28.2).

Surface IgA-expressing B cells are usually low in patients with IgAD, and most cells express an immature phenotype. However, the proportion of surface Ig-positive cells remains unaffected in a majority of CVID patients. These B cells do not mature properly into antibody-producing cells and plasma cell numbers are low in the bone marrow. A large number of cell surface CD markers have been analyzed in IgAD and CVID. Although their diagnostic value is limited in patients with IgAD, reduction of switched memory B cells ($CD27^+$ cells) and an expansion of $CD21(low)^+$ B cells has been suggested to serve as a basis for subclassification of CVID patients (Wehr et al., 2007).

IgAD is probably manifest already at birth in a majority of cases, and the prevalence in 4-year-old Swedish children is approximately 1 in 200 (Janzi et al., 2009), as compared to 1 in 600 adult Swedes. IgA production in most children matures gradually, and at 8 years of age, half of the children who were IgAD at 4 years of age have normal IgA levels. However, induction of IgAD has also been described in a number of children with celiac disease.

Antibodies against IgA are typical markers of IgAD and CVID, and these autoantibodies are not seen in individuals with normal levels of IgA.

GENETIC COMPONENT OF IgAD AND CVID

HERITABILITY OF IGAD AND CVID

Most cases of IgAD and CVID described to date have been sporadic, but familial cases have been observed, and a susceptibility trait can occasionally be traced back for many generations (Wollheim et al., 1964). Thus far, more than 100 families with multiple cases of IgAD and/or CVID have

Table 28.2 CHARACTERISTIC LABORATORY FINDINGS IN IGAD AND CVID

PARAMETER	CVID	IGAD
Serum IgM levels	Low/normal	Normal
Serum IgG levels	Low	Normal/high
Serum IgA levels	Low	Low
Secretory IgA	Low	Low
Surface IgM-positive cells	Normal	Normal
Surface IgG-positive cells	Normal	Normal
Surface IgA-positive cells	Normal	Low/normal
Autoantibodies (anti-IgA)	10–20%	20–40%
Lymphocyte CD markers	Altered	Normal
Mitogen responsiveness	Low/normal	Normal
Plasma cells (bone marrow)	Low	Normal

been reported in the literature (for references see Kralovicova et al., 2003; Vorechovsky et al., 1995), and the mode of inheritance of the deficiency has been suggested to be either recessive or dominant. In 20 percent of the published cases, both IgAD and CVID occurred in the same family, usually presenting with CVID in the parental generation and IgAD in the children.

The human leukocyte antigens (HLA), located within the MHC region on chromosome 6, are implicated in the genetic susceptibility to a large number of diseases, and as early as the late 1970s, an association between IgAD and certain HLA types was suggested. In the early 1980s, it was shown that there was indeed an association between isolated IgA deficiency and the MHC region (Oen et al., 1982), in particular the HLA class II region (Hammarstrom and Smith, 1983). A genetic predisposition, similar to that found in IgA deficiency, has also been observed in patients with CVID (Olerup et al., 1992; Schaffer et al., 1989). However, the latter appears to be restricted to a rather small subgroup of patients, and most CVID patients show no apparent HLA association.

In spite of intense research during the past decades, the susceptibility genes within the HLA region have not been identified (for recent review on MHC association of IgAD see Rioux et al., 2009). Several reports have suggested that distinct loci in the class II and class III region, located on different MHC haplotypes, confer susceptibility to development of IgAD (de la Concha et al., 2002; Gual et al., 2004; Kralovicova et al., 2003; Olerup et al., 1990, 1991; Schroeder et al., 2004).

Recently, we observed an association with several non-MHC genes in patients with IgAD (Ferreira et al., 2010). These susceptibility genes, including IFIH1 and CLEC16A, are also found in patients with "classical" autoimmune diseases. In this respect, it is worth noting that IgAD is also more common among patients with type 1 diabetes, SLE, and celiac disease, suggesting a genetic link between immunodeficiency and susceptibility to autoimmunity.

Monogenic Defects Underlying IgAD/CVID

The identification of a homozygous mutation in the inducible co-stimulation (*ICOS*) gene in four patients from two unrelated families from the Black Forest with CVID in 2002 was a major breakthrough in CVID research (Grimbacher et al., 2003). To date, five additional patients with the originally described large deletion in the *ICOS* gene have been identified (Yong et al., 2009), as have two Japanese patients with a unique *ICOS* deletion (Takahashi et al., 2009). ICOS belongs to the CD28 family of co-stimulatory molecules and is expressed exclusively on activated T cells. It has at least three major functions: germinal-center formation, isotype class switching, and the development of memory B cells. Recently, a critical role for ICOS in the development of human TH17 cells has also been suggested (Paulos et al., 2010). The discovery of human ICOS deficiency showed, for the first time, that a monogenic disorder could account for the full spectrum of manifestations seen in childhood- and adulthood-onset CVID, including autoimmune, inflammatory, and malignant disease complications, as well as recurrent infections.

TACI and BAFFR are TNF-like receptors, responding to a number of ligands (BAFF, BCMA, and APRIL) involved in maturation and differentiation of B cells. In view of the impaired Ig class switching described in APRIL- (Castigli et al., 2004) and BAFF-deficient mice (Schiemann et al., 2001), it seems likely that similar defects, involving this family of receptors/ligands, may be involved in the pathogenesis of selected cases of CVID in man. Indeed, truncating and missense mutations have been described both in TACI (*TNFRSF13B*) (Salzer et al., 2005, 2008) and BAFFR (*TNFRSF13C*) (Warnatz et al., 2009) and shown to be associated with a CVID phenotype. The former may account for up to 10 percent of all patients, whereas the latter seems much less frequent.

The CD19 molecule expressed by B lymphocytes and follicular dendritic cells of the hematopoietic system is the earliest of B-lineage restricted surface antigens. It is expressed on the cell surface associated with CD21, CD81, and CD225, forming the B-cell receptor complex. The CD19 molecule is involved in development of B1 and marginal-zone B cells; when lacking, antibody responses and B-cell memory are strongly affected. Mutations in *CD19* in patients with CVID have recently been described in three separate families (Kanegane et al., 2007; van Zelm et al., 2006). It is likely, however, that this is a very rare cause of CVID.

MSH5, a gene encoded in the central MHC class III region, and its obligate heterodimerization partner Msh4 play a critical role in regulating meiotic homologous recombination. Genetic variation in *MSH5* has been shown to be associated with IgAD and CVID in humans (Sekine et al., 2007) One of the human MSH5 alleles identified contains two nonsynonymous polymorphisms, where the variant protein shows impaired binding to Msh4. Ig switch region joints from CVID and IgAD patients carrying the disease-associated *MSH5* alleles show increased donor/acceptor microhomology, involving pentameric DNA repeat sequences and lower mutation rates than controls. Thus, Msh4/5 heterodimers may contribute to class-switch recombination (CSR) where

Msh4/5 promotes the resolution of DNA breaks with low or no terminal microhomology.

CD81 is complexed with CD19, CD21, and CD225, which together form the B-cell receptor. A patient was recently reported with severe nephropathy and profound hypogammaglobulinemia characterized by decreased memory B-cell numbers, impaired specific antibody responses, and an absence of CD19 expression on B cells (van Zelm et al., 2010). The patient carried a homozygous *CD81* mutation resulting in a complete lack of CD81 expression on leukocytes. Similar to CD19-deficient patients, the CD81-deficient patient had B cells that showed impaired activation upon stimulation via the B-cell antigen receptor but no overt T-cell defects.

A recently identified 28-year-old man with hypogammaglobulinemia, recurrent respiratory infections, diarrhea, and splenomegaly was found to have a compound heterozygous deleterious mutation in the *CD21* gene, which encodes the complement receptor (CD21) expressed by B cells as part of the B-cell receptor. Functional studies of patient B cells showed complete loss of co-stimulatory activity of C3d-containing immune complexes. B-cell numbers as determined by CD19 and CD81 were normal, but the percentage of class-switched memory B cells was markedly reduced. Antibody responses to pneumococcal polysaccharide antigens were abnormal, while antibody responses to protein antigens were normal (Thiel et al., 2011).

Absence of CD27, a marker for memory B cells, was recently observed in two brothers with homozygous mutation in the *CD27* gene (van Montfrans et al., 2012). In addition to hypogammaglobulinemia, abnormal antibody responses to T-dependent antigens, and T-cell dysfunction, both patients suffered from persistent symptomatic Epstein-Barr virus (EBV) viremia, with one of the two brothers developing aplastic anemia thought to be caused by EBV, leading to fulminant gram-positive sepsis and the death of one brother at age 4 years. The surviving brother, now 21 years old, became progressively hypogammaglobulinemic and developed hepatosplenomegaly. He is responding well to regular IVIG infusions.

Mutations in *CD20* (Kuijpers et al., 2010) have recently also been suggested to cause a mild form of hypogammaglobulinemia, yet not fulfilling the criteria of CVID.

FUNCTIONAL ASPECTS

DEFECTIVE IG CLASS SWITCH IN IGAD AND CVID

Ig isotype switching is preceded by transcription of the germline mRNA for immunoglobulins (I) and switch regions upstream of the Ig heavy-chain constant-region gene, generating germline transcripts. Germline Iα transcripts are easily detected in unstimulated peripheral blood mononuclear cells from normal donors, whereas they are absent in cells from IgAD individuals (Islam et al., 1994). However, α-containing transcripts can be readily induced by the addition of TGF-β, demonstrating that the locus is accessible for transcription provided that the required signals are supplied.

A cell culture system that allows the expression of immunoglobulins by cells from IgAD and CVID patients has previously been described (Borte et al., 2009; Briere et al., 1994; Zielen et al., 1993). This system utilizes anti-CD40 antibodies or CD40L in conjunction with interleukin (IL)-4, IL-10, or IL-21, demonstrating that secretion of IgA can indeed be achieved if appropriate stimuli are added. However, although the timing and concentration of cytokines may play a crucial role in the switching process, there is as yet no strong evidence to implicate any cytokine or cytokine receptor as playing a role in the disease process.

DRUG-INDUCED IG DEFICIENCIES

IgA deficiency is seen, although infrequently, in patients treated with a variety of antirheumatic and antiepileptic drugs (Table 28.3). CVID and IgG2-IgA deficiency is also induced by some of the implicated drugs. Selected individuals may be genetically predisposed to develop drug-induced Ig deficiency, as multiple drugs can induce immunodeficiency in a given patient (Farr et al., 1991). In approximately half of the reported patients the deficiency has been reversible after cessation of treatment, although full recovery requires months or even years.

The mechanism underlying the drug-induced form of IgAD and CVID has not been determined, and there is as yet no common molecular denominator in the drugs used.

STRATEGIES FOR DIAGNOSIS

Selective IgAD presents hardly any diagnostic problems in adults, but as serum IgA levels may remain low for a protracted period in children (Janzi et al., 2009; Plebani et al., 1986), a definite diagnosis of IgAD cannot be made before the teens, although the concomitant absence of IgG2 and IgG4, even in early childhood, is a strong indicator of persistent IgAD.

Surface Ig-positive cells remain unaffected in the blood in a majority of CVID patients. However, in patients with XLA, these cells are low or absent, which may aid in differential diagnosis in young boys. The latter could also be identified by demonstrating absence of BTK or by mutation screening. The spectrum of infections also differs slightly, and some patients with CVID are also prone to nonbacterial infections as a subset of patients show a concomitant T-cell defect.

A tentative distinction between CVID and the class-switch recombination defects can be made based on sex, as most patients show an X-linked inheritance pattern. Again, the spectrum of infections appears slightly different, and as most implicated genes involved have been cloned (see Chapters 26 and 27), a correct diagnosis is possible by mutation screening.

X-linked lymphoproliferative disease (XLP) is a primary immunodeficiency caused by a defect in the *SH2D1A*, *XIAP*, or *MAGT1* gene (Al-Herz et al., 2011). The disease is characterized by severe, often fatal infectious mononucleosis, lymphomas, and Ig deficiencies. Although patients with "typical" cases (see Chapter 44) are easily distinguishable from CVID patients, problems in establishing the differential diagnosis may

Table 28.3 PATTERN OF DRUG-INDUCED IMMUNOGLOBULIN DEFICIENCIES

DRUG	CVID	IgGD-IgAD	IgAD
Sulfasalazine	X	X	X
Gold			X
Chloroquine			X
Penicillamine			X
Captopril			X
Fenclofenac			X
Hydantonin	X	X	X
Carbamazepine	X		X
Valproate			X
Thyroxine[a]			X
Levamisol[a]	X		
Ibuprofen[a]			X
Salicylic acid[a]			X
Cyclosporin A[a]			X

[a] Requires independent confirmation

occur (Morra et al., 2001, Soresina et al., 2002), and sequencing of the implicated genes may therefore be warranted.

The WHIM (warts, hypogammaglobulinemia, immunodeficiency, myelokathexis) syndrome, due to mutations in the chemokine receptor *CXCR4* gene (see Chapter 40), represents yet another potential, although rare, differential diagnosis to CVID. Again, sequencing of the implicated gene might be required.

In patients with hypogammaglobulinemia due to thymoma (Good's disease), B cells are lacking altogether in both peripheral blood and in the bone marrow, and a scan of the thymus may indicate the presence of a thymoma. Age at onset may also be of some help in the differential diagnosis of hypogammaglobulinemia. Adult onset of infections clearly suggests CVID rather than XLA, and thymoma-affected patients are usually, although not invariably, older than the average CVID patient.

TREATMENT OPTIONS

The foundation of therapy for CVID and IgAD patients is antibiotics and Ig replacement. Gammaglobulin infusions have been used for decades in patients with CVID and in infection-prone patients with IgAD (Gustafson et al., 1997). The doses employed have gradually risen, and today a weekly dose of 100 to 150 mg/kg of body weight is recommended.

Prior to being introduced as replacement therapy for XLA patients, Ig had been given intramuscularly as prophylaxis against various viral diseases. Bruton (1952), however, started his agammaglobulinemic patients on prophylactic treatment using subcutaneous Ig injections, a treatment regimen later altered to intramuscular administration. The intramuscular injections remained the mode of therapy for immunodeficiency patients for almost two decades, but owing to the limited amount that could be given, IVIG preparations were subsequently developed.

Berger et al. (1980) reintroduced treatment with slow subcutaneous infusions, and a number of reports on the treatment of hypogammaglobulinemic patients followed (for references, see Gardulf et al., 1991). However, due to the slow rate of infusion, this form of treatment did not meet with widespread appreciation.

Home treatment of patients with primary immunodeficiency disorders using gammaglobulin for intravenous use (IVIG) was introduced in 1986 (Ochs et al., 1986) and represented a significant step forward in replacement therapy. This initial report was soon followed by additional reports (Ashida and Saxon, 1986; Chapel et al., 1988; Ryan et al., 1988) showing the effectiveness of the method.

Rapid subcutaneous infusion of Ig preparations intended for intramuscular administration was first described by Gardulf et al. (1991). The method can be used in patients with a previous history of adverse reactions even in a home setting, does not require the presence of a trained partner, and results in a very low incidence of adverse reactions (Gardulf et al., 1995, 2007). Thus, this method currently represents an attractive mode of Ig administration.

Daily self-administered subcutaneous replacement therapy using small doses of gammaglobulin has recently been introduced as therapy for selected patients (Shapiro, 2010). The opposite regimen (i.e., prolonging the intervals between injections) has also been attempted, where subcutaneously administered gammaglobulin is given every second week (Gustafson et al., 2008).

In spite of replacement therapy with intravenous or subcutaneously administered Ig, some patients still suffer from respiratory and gastrointestinal tract infections. Topical application of Ig preparations (nasal, ocular, oral) has therefore been tried with some therapeutic success in selected patients.

ANIMAL MODELS

Although there are a number of reports of IgAD dogs (Felsburg et al., 1985; Glickman et al., 1988; Moroff et al., 1986) and chickens (Luster et al., 1976), the molecular basis of these deficiencies has not been elucidated. However, owing to recent progress in the field of canine genetics, dogs may well be a suitable system for searching for immunodeficiency-associated genes (Karlsson and Lindblad-Toh, 2008; Wilbe et al., 2010).

There is not as yet a rodent model available that resembles the human disease, although knockout mice with a deleted J chain (Hendrickson et al., 1995), Iα region (Harriman et al., 1996), or Cα region (Harriman et al., 1999) have been described. In the J chain-deficient mice, only secretion of IgA is impaired, and serum levels of IgA are up to 30-fold higher than in normal wild-type mice. Interestingly, there may be a J chain-independent IgA transport in the intestinal, mammary,

and respiratory epithelial cells of these mice (Hendrickson et al., 1996), adding to the complexity of the "secretory" IgA machinery. Mice with an I-region deletion produce normal levels of IgA. This observation suggests that the I region as such is redundant and can be replaced by other gene sequences, thus suggesting that splicing of germline transcripts rather than transcription per se controls DNA rearrangement leading to class switch.

Mice with a targeted inactivation of the α heavy chain constant region gene as well as part of the Sα region have also been produced (Harriman et al., 1999). These mice are profoundly deficient in both systemic and secretory IgA and show a perturbed pattern of expression of immunoglobulins, manifested as an increased level of IgM and IgG (primarily IgG2) and decreased levels of IgG3 and IgE both in serum and secretions. These mice are, however, largely normal with respect to development of their lymphoid tissues and proliferative responses. Cytokine profiles in response to antigenic challenge (influenza virus) are, however, altered, with a downregulation of Th1-mediated immune responses (Zhang et al., 2002).

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29.

INTRODUCTION TO SYNDROMES OF IMMUNE DYSREGULATION AND AUTOIMMUNITY

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Immunodeficiency disorders have traditionally been thought of as conditions resulting from the inability to mount effective host responses to infectious agents. However, immune dysregulation and autoimmune phenomena are seen in many inherited and acquired immunodeficiency disorders. Particular alleles of the major histocompatibility complex (MHC) locus have long been associated with increased susceptibility to a variety of diseases with an autoimmune basis—for example, insulin-dependent diabetes mellitus and celiac disease (Bodmer, 1996; Hammer et al., 1997; Thorsby, 1997). The detailed mechanisms that allow tolerance to be broken so that immune reactions are directed against one's own tissues are becoming better understood. The composition of the lymphocyte antigen recognition repertoire and the potential for cross-reactivity between self antigens in host tissues and foreign antigens from the environment is defined in part by an individual's MHC haplotype. With improved understanding of the processes directing selection, expansion, and control of an appropriate lymphocyte repertoire, several non-MHC-linked heritable immune disorders are now recognized in which disruption of tolerance and/or regulatory functions is a predominant feature. The chapters in this section highlight how autoimmune phenomena are an important aspect of primary immunodeficiency disease (PIDD). The group of single-gene disorders discussed in Chapters 30 through 33 are characterized by susceptibility to immune dysregulation and autoimmunity. While the typical findings commonly listed for primary disorders of the immune system relate to recurrent or unusually severe infections, autoimmune complications are seen in many PIDD settings. Indeed, for patients with some of the known defects in adaptive or innate immunity, autoimmunity and immune dysregulation are the predominant findings.

Dysregulation of adaptive immune responses can interfere with the central and peripheral control of self-reactive T cells, resulting in severe, life-threatening autoimmune phenomena, independent of any known infection (Westerberg et al., 2008). Under normal conditions, autoreactive T cells, generated in the thymus, are eliminated by negative selection: when the T-cell receptor (TCR) of a newly formed thymic T cell strongly recognizes an MHC molecule loaded with a self-antigen peptide, such as insulin, this autoreactive T cell receives a death signal and is promptly deleted by apoptosis. This process is controlled by the autoimmune regulator (*AIRE*) gene, with codes for a transcription factor expressed in medullary thymic epithelial cells. AIRE induces mature thymic epithelial cells to express and present to nascent T cells a variety of tissue restricted antigens representing organs throughout the body. The resulting deletion of the autoreactive T cells is referred to as *central tolerance*. When *AIRE* is mutated, as in autoimmune polyglandular syndrome type 1 (APS1), also called autoimmune polyendocrinopathy, candidiasis, ectodermal dysplasia (APECED), this central tolerance is impaired, resulting in a diverse spectrum of autoimmune disorders (Chapter 31).

$CD4^+$ $CD25^+$ $FOXP3^+$ natural regulatory T cells (nTreg), although generated mainly in the thymus, eventually exit to the periphery and ensure that self-reactive T cells that have escaped the central tolerance control system in the thymus are nonetheless prevented from attacking one's own tissues (peripheral tolerance). If the transcription factor FOXP3, encoded on the X chromosome, is missing or defective, nTregs fail to develop, resulting in the syndrome known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) (Chapter 32).

It is not surprising that single-gene defects resulting in reduced numbers or decreased fitness of nTregs are associated

with syndromes characterized by autoimmunity. These disorders include deficiency of CD25 (Chapter 32), a component of the high-affinity IL-2 receptor that is expressed by Tregs; defective STAT5b (Chapter 32), a transcription factor involved in the regulation of FOXP3 expression; and impaired or absent Wiskott-Aldrich syndrome protein (WASp), a protein required for synapse formation between Tregs and effector T cells (Chapter 43).

A third group of T-cell deficiencies associated with autoimmunity are caused by defective T-cell development due to impaired VDJ rearrangement, in which limited diversity of TCR rearrangement in the thymus leads to clones of self-reactive T cells, as in DiGeorge and Omenn syndromes, the latter frequently caused by hypomorphic mutations in RAG1, RAG2, or other proteins (Artemis, Ligase4) required for TCR rearrangement (Chapter 13).

Autoantibodies are directly involved in the pathogenesis of antibody-mediated cytopenias and several other autoimmune diseases, indicating the importance of autoreactive B cells. Analogous to the establishment of T-cell tolerance in the thymus, early B-cell progenitors include a high proportion of self-reactive cells, which are effectively purged in the bone marrow and later in the periphery by receptor editing and other mechanisms that remove autoreactive B cells using central and peripheral B-cell tolerance checkpoints (Meffre and Wardemann, 2008).

Apoptosis is an important mechanism for elimination of autoreactive lymphocytes, and defective apoptosis due to dominant germline or somatic mutations in the apoptosis receptor Fas cause autoimmune lymphoproliferative syndrome (ALPS) (Chapter 30). Additional apoptosis mediators have likewise been implicated in conditions in which T cells fail to die when they should, leading to persistence of elevated numbers of $CD4^-CD8^-$ mature $CD3^+$ T cells (which express α,β T-cell receptors), autoimmune blood cytopenias and other autoimmune disorders, and elevated risk of developing lymphoma (Turbyville and Rao, 2010).

Patients with chronic granulomatous disease (CGD) are prone to autoimmune complications such as sarcoidosis and Crohn's disease (Chapter 52). This may be due to persistent inflammation caused by secretion of proinflammatory cytokines (TNFα, IL-1β, IL-6) and/or accumulation of microorganism-derived antigens in tissue macrophages. Defective apoptosis of CGD neutrophils, as suggested by diminished surface expression of phosphatidyl serine (PS), may contribute to the delayed removal of antigen-loaded neutrophils by macrophages and, as a consequence, the production of autoantibodies resulting in discoid or systemic lupus.

Finally, congenital defects in the early complement components (C1q, C1r/s, C4, and C2) (Chapter 55) are strongly associated with systemic lupus erythematosus, demonstrating the need for an intact classical complement pathway for the safe elimination of immune complexes and prevention of immune complex disease.

Inherited susceptibility to autoimmunity associated with immune deficiency poses diagnostic and therapeutic challenges, affects the patient's long-term prognosis, and has implications for family members who may be at risk themselves or may have an increased chance of having affected offspring.

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30.

AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

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Apoptosis, or programmed cell death, is a critical process for deleting lymphocytes with self-reactive potential, both during lymphocyte development and in the peripheral blood and tissues throughout life. After leaving the thymus, mature T cells normally undergo a life cycle of activation and effector responses followed by apoptosis. Apoptosis maintains immune homeostasis and serves as one mechanism to minimize potential reactions against self-antigens by limiting lymphocyte accumulation after appropriate expansion in response to antigenic challenge. It is not surprising that a process as important as apoptosis is tightly regulated by interactions among multiple components. Two major mechanisms for the death of lymphocytes after their stimulation are recognized (Lenardo et al., 1999). Passive (intrinsic) apoptosis mediated by mitochondrial mechanisms follows cytokine (e.g., interleukin [IL]-2) withdrawal after clearance of antigen, while active (extrinsic) apoptosis requires the engagement of specific cell-surface molecules, principally the FAS receptor, a member of the tumor necrosis factor receptor gene superfamily also known as CD95 or APO-1.

After lymphocytes have been stimulated and are proliferating, a second or prolonged encounter with antigen induces expression of both the transmembrane receptor FAS and its ligand, FAS ligand (FASL). Extracellular engagement of a trimer of FASL chains with homotrimeric Fas receptor triggers a cascade of intracellular molecular events leading to alterations in the cell membrane, DNA fragmentation, and ultimately the death of the cell. An animal model that reveals the in vivo importance of this pathway in maintaining lymphocyte homeostasis is illustrated by the lymphoproliferation, autoantibody formation, and autoimmune nephritis that develops in mice with *lpr* and *gld* mutations, genetic defects in Fas and FasL expression, respectively (Nagata and Goldstein, 1995). In these models there are factors beyond the primary mutation that modify disease as evidenced by differences in the severity of autoimmunity between various strains. The relevance of this apoptotic pathway to human disease was postulated in 1992 with a description of two children with massive, nonmalignant lymphoid hyperplasia and autoimmune disease (Sneller et al., 1992). Subsequently, Rieux-Laucat et al. (1995), from INSERM in Paris, and Fisher et al. (1995), from the U.S. National Institutes of Health (NIH), documented that this disorder, now called autoimmune lymphoproliferative syndrome (ALPS), is associated with inherited mutations in the gene encoding FAS, *FAS* (also called *TNFRSF6*, for tumor necrosis factor receptor superfamily member 6). Previous descriptions of patients with a clinical condition consistent with ALPS include pseudolymphoma and Canale-Smith syndrome (Canale and Smith, 1967; Rao et al., 1974). Further reports by these and other teams of investigators have enlarged the number of known patients with ALPS to over 500 and documented that the syndrome can be associated with a broad clinical spectrum and molecular defects in apoptosis pathway members other than the transmembrane FAS receptor. While the series of patients followed at NIH is currently the largest (Bleesing et al., 2001; Caminha et al., 2010; Jackson et al., 1999; Sneller et al., 1997, 2003), patients have been reported worldwide (Bettinardi et al., 1997; Drappa et al., 1996; Le Diest et al., 2003; Rieux-Laucat et al., 2003; Seif et al., 2010), providing an increasingly comprehensive picture of the clinical, genetic, and immunological features of ALPS and its consequences for a patient bearing this defect in lymphocyte apoptosis present over a period of decades.

Even among the cohorts of ALPS patients bearing *FAS* mutations, the story is a complex one. The majority of patients with ALPS have inherited germline, dominant, heterozygous *FAS* mutations, but compound heterozygous

Table 30.1 **DIAGNOSTIC CRITERIA FOR ALPS***

I. Required
Chronic (>6 months duration) nonmalignant, noninfectious lymphadenopathy and/or splenomegaly
Elevated numbers of $CD3^+$ $TCR\alpha/\beta^+$ $CD4^-$ $CD8^-$ DNT cells (≥1.5% of total lymphocytes or 2.5% of $CD3^+$ lymphocytes) in the setting of normal or elevated lymphocyte counts
II. Accessory
Primary
1. Defective lymphocyte apoptosis (in two separate assays)
2. Somatic or germline pathogenic mutation in *FAS*, *FASL*, or *CASP10*
Secondary
1. Elevated plasma sFASL levels (>200 pg/mL) OR elevated plasma IL-10 levels (>20 pg/mL) OR elevated serum or plasma vitamin B12 levels (>1,500 ng/L) OR elevated plasma IL-18 levels (>500 pg/mL)
2. Typical immunohistological findings as reviewed by an experienced hematopathologist
3. Autoimmune cytopenias (hemolytic anemia, thrombocytopenia, or neutropenia) AND elevated IgG levels (polyclonal hypergammaglobulinemia)
4. Family history of a nonmalignant, noninfectious lymphoproliferation with or without autoimmunity

Definitive diagnosis ALPS: both required criteria plus one primary accessory criterion

Probable diagnosis ALPS: both required criteria plus one secondary accessory criterion

or homozygous mutations have been found in a few severely affected patients with early-onset disease starting in infancy. In addition, somatic *FAS* mutations have now been identified as the second most commonly defined molecular basis of ALPS (Dowdell et al., 2010; Holzelova et al., 2004). Most of the mutant *FAS* alleles from patients with ALPS have a dominant negative effect on apoptosis by normal alleles co-expressed in the same cell. The relatives of ALPS patients who share the same FAS defects may have mild or undetectable lymphoproliferation and autoimmunity, or may be severely affected. Taken together, these findings suggest that factors in addition to heterozygous defects of FAS are required for the full clinical expression of ALPS, similar to observations in the murine models. In addition, individuals with inherited FAS defects are at increased risk of lymphoma and possibly other malignancies (Rao et al., 2010; Straus et al., 2001). Thus, as the first human immune disorder to be associated with defective lymphocyte apoptosis, ALPS clarifies the critical role of cell death in health and disease.

DIAGNOSTIC CRITERIA AND CLASSIFICATION BY GENOTYPE

ALPS is defined as an impairment of lymphocyte apoptosis that includes chronic, nonmalignant, noninfectious lymphadenopathy and/or splenomegaly, plus expansion of a normally rare subset of lymphocytes in the peripheral blood and tissues—$CD3^+$ T cells that express α/β T-cell receptors ($TCR\alpha/\beta^+$) but express neither CD4 nor CD8 co-receptors ($CD4^-$ $CD8^-$), cells that are referred to as double-negative T (DNT) cells. The recently revised case definition of ALPS (Table 30.1) requires nonmalignant, noninfectious lymphadenopathy and/or splenomegaly persistent for at least 6 months and DNT expansion along with either defective lymphocyte apoptosis as assayed in vitro (on at least two occasions) or a defined disease-associated mutation in *FAS*, *FASL* (also called *TNFSF6*) or *CASP10* (Oliveira et al., 2010). This classification system also includes a probable ALPS category that includes the two required findings noted above together with one associated abnormality: elevated ALPS biomarker levels (soluble FASL [sFASL], IL-10, IL-18, or vitamin B12), typical immunohistological findings on lymph node biopsy, autoimmune cytopenia with hyperglobulinemia, or a family history of a similar clinical condition (Oliveira et al., 2010). The original classification system for genotypes of ALPS established by the NIH group (Puck and Straus, 2004), with variations suggested by others (Holzelova et al., 2004; Rieux-Laucat et al., 2003), has recently been revised based on an international consensus conference (Oliveira et al., 2010). As shown in Table 30.2, ALPS-FAS includes the originally reported patients, in whom germline (homozygous or heterozygous) *FAS* mutations produce deleterious consequences for the expression or function of the FAS protein. This type accounts for two thirds to three quarters of all ALPS cases in various series, with the majority having dominant, heterozygous *FAS* mutations, but rare patients with compound heterozygous mutations and a patient with homozygous null mutation of *FAS* have been reported (Jackson et al., 1999; Rieux-Laucat et al., 1995; van der Burg et al., 2000). Patients with somatic mutations in *FAS* (Dowdell et al., 2010; Holzelova et al., 2005) are designated as ALPS-sFAS and are now known to represent the second largest ALPS patient group with a defined genetic basis for disease.

Patients who fulfill the case definition for ALPS based on an abnormal in vitro FAS-mediated apoptotic defect but who do not have a defined genetic mutation (including a somatic *FAS* mutation) are referred to as having ALPS-U (unknown). Patients with a defect in *FASL* as initially described in an adult patient with atypical lupus are defined

Table 30.2 REVISED CLASSIFICATION OF ALPS*

REVISED NOMENCLATURE	PREVIOUS NOMENCLATURE	GENE	DEFINITION
ALPS-FAS	ALPS type 0	*FAS*	Patients fulfill ALPS diagnostic criteria & have germline homozygous *FAS* mutation
ALPS-FAS	ALPS type 1a	*FAS*	Patients fulfill ALPS diagnostic criteria & have germline heterozygous *FAS* mutation
ALPS-sFAS	ALPS type 1m	*FAS*	Patients fulfill ALPS diagnostic criteria & have somatic *FAS* mutation
ALPS-FASL	ALPS type 1b	*FASL*	Patients fulfill ALPS diagnostic criteria & have germline *FASL* mutation
ALPS-CASP10	ALPS type IIa	*CASP10*	Patients fulfill ALPS diagnostic criteria & have germline *CASP10* mutation
ALPS-U	ALPS type III	Unknown	Patients fulfill ALPS diagnostic criteria but genetic defect is undetermined (no *FAS*, *FASL*, or *CASP10* defect)

* Oliveira et al., 2010

as having ALPS-FASL (Wu et al., 1996), and patients with a defect in *CASP10* are now classified as having ALPS-CASP10 (Wang et al., 1999; Zhu et al., 2006). Patients who meet the two primary clinical and laboratory criteria for ALPS without mutations in apoptosis pathway genes or a demonstrable apoptotic defect but in whom an additional supporting finding is present are now referred to as having probable ALPS (Oliveira et al., 2010).

Two siblings who presented with a phenotype that included certain ALPS features plus immunodeficiency that is associated with a defect in caspase 8 encoded by the *CASP8* gene are now classified as having an ALPS-related condition referred to as caspase 8 deficiency state (CEDS) (Table 30.3; Chun et al., 2002). Additional patients have been described who have some features of ALPS but no demonstrable in vitro defects of apoptosis or mutation in apoptosis pathway components; these patients are also included in the ALPS-related disorder category, with a designation of DALD (Dianzani-associated lymphoproliferative disorder) in Table 30.3 (Dianzani et al., 1997). Finally, more recently described patients have been identified with an ALPS-related disorder associated with a somatic gain of function mutation in either the *NRAS* or *KRAS* genes. This constellation of findings results in a defect affecting the intrinsic apoptotic pathway induced by cytokine withdrawal and is now categorized as RAS–associated autoimmune leukoproliferative disease (RALD) (Table 30.3; Niemela et al, 2010; Oliveira et al., 2007).

EPIDEMIOLOGY

ALPS has been diagnosed in both males and females of diverse racial backgrounds, but at this juncture no figures estimating incidence are available. However, patients with idiopathic lymphoproliferation and autoimmune phenomena, sometimes inherited as autosomal dominant traits, have appeared in multiple literature reports and clinical series from the 1960s onward (Canale and Smith, 1967; Cheng et al., 1980; Holimon and Madge, 1971; Randall et al., 1965; Rao et al., 1974). Some of these individuals are likely to have been suffering from ALPS, and in at least three cases ALPS has been proven in follow-up studies with the detection of mutations in *FAS* (Drappa et al., 1996; and NIH unpublished data). Patients with medical findings consistent with ALPS have been under the care of physicians in many specialties: hematologists, called upon to manage the thrombocytopenia and hemolytic anemia; oncologists, who have biopsied the enlarged lymph nodes and sometimes felt compelled to treat patients with persistent adenopathy as if they had lymphoma; and rheumatologists, who have evaluated and managed some of their autoimmune phenomena.

In patients with defined mutations of *FAS* it has been possible to recognize multiple relatives bearing identical mutations but with highly variable clinical penetrance. Similar mutations have not been detected in significant numbers of healthy individuals screened at random. In some

Table 30.3 REVISED CLASSIFICATION OF ALPS-RELATED DISORDERS*

REVISED NOMENCLATURE	PREVIOUS NOMENCLATURE	GENE	DEFINITION
Caspase 8 deficiency state (CEDS)	ALPS type IIb	*CASP8*	Patients present with lymphadenopathy and/or splenomegaly, DNT elevation, recurrent infections, and germline *CASP8* mutation
RAS-associated autoimmune leukoproliferative disorder (RALD)	ALPS type IV	*NRAS*	Patients present with autoimmunity, lymphadenopathy and/or splenomegaly, elevated or normal DNTs, and somatic *CASP8* mutation
Dianzani autoimmune lymphoproliferative disease (DALD)	DALD	Unknown	Patients present with autoimmunity, lymphadenopathy and/or splenomegaly, normal DNTs, and defective in vitro FAS-mediated apoptosis

* Oliveira et al., 2010

Table 30.4 CLINICAL FINDINGS IN PROBANDS WITH ALPS-FAS AND THEIR *FAS*-MUTATION-POSITIVE RELATIVES (NIH COHORT)

CLINICAL FINDING	PROBANDS* (N = 79)	RELATIVES† (N = 164)
AGE OF DIAGNOSIS	BIRTH–15 Y (MEAN 2 Y)	NOT AVAILABLE
SEX	36 M, 43 F	
Lymphoproliferation	100%	44%
Lymphadenopathy	92%	30%
Splenomegaly	88%	23%
Splenectomy	51%	17%
Hepatomegaly	72%	Not available
Autoantibodies in significant titer	69%	24%
Overt autoimmune disease	70%	24%
Hemolytic anemia, Coombs-positive	51%	12%
Idiopathic thrombocytopenic purpura	47%	12%
Neutropenia	23%	2%
Hepatitis, biliary cirrhosis	2–3%	1–2%
Optic neuritis, uveitis, episcleritis	2–3%	0%
Thyroiditis, hypothyroidism	1–2%	2%
Glomerulonephritis	1–2%	0%
Polyneuroradiculitis (Guillain-Barré)	1%	1–2%
Encephalomyelitis	1–2%	0%
Skin rashes, including urticaria, vasculitis	Common	NA
Malignancy		
Lymphoma	9%	4%

*Proband, the first member of each family referred to NIH.

†While some relatives with *FAS* mutations meet the criteria for the full definition of ALPS, others do not due to reduced penetrance and variable expressivity.

ALPS families, most or all individuals with *FAS* mutations meet the strict criteria for ALPS, as listed in Table 30.1, while other genetically affected persons can be found with few or no clinical, immunological, or hematological manifestations.

CLINICAL MANIFESTATIONS OF ALPS

Clinical features of ALPS-FAS are listed in Table 30.4. The frequencies of each feature are given for probands, defined as unrelated individuals who were the first in their kindred to be referred to NIH, and their relatives who carry the same *FAS* mutations as the probands (Jackson et al., 1999; Sneller et al., 2003; and NIH unpublished data). Other series are similar in overall range and frequency of clinical features (Le Deist et al., 1996, Vaishnaw et al., 1999a). The presentation of probands with splenomegaly and adenopathy is most often in infancy or early childhood, with a mean age of 2 years; only rarely do patients present with adenopathy as late as 15 years. At least three children were clinically affected at birth (Bettinardi et al., 1997; Le Diest et al., 1996, 2003).

LYMPHOPROLIFERATION

Lymphoproliferation is the most dramatic and consistent clinical feature of ALPS (Table 30.4). In addition, patients almost always develop splenomegaly of moderate to massive proportions before age 5. Although the dimensions of the spleen may fluctuate over time in a given patient, palpable splenomegaly almost always persists. The spleen may extend only 2 to 4 cm below the left costal margin or past the umbilicus and into the pelvis with commensurate degrees of abdominal distention. Splenectomy, originally performed in the majority of patients to manage blood cytopenias, is now strongly discouraged unless severe hypersplenism is refractory to other measures due to the increased risk of systemic bacterial infection after splenectomy. Splenectomy has been necessitated in ALPS patients by traumatic rupture of their enlarged spleens.

Mild to moderate hepatomegaly is observed in most patients at some point in time. However, clinical signs of liver dysfunction are uncommon in the absence of hepatitis C virus infection, which has complicated the course of some patients who have received blood transfusions for management of severe hemolytic anemia.

Lymphadenopathy is a consistent feature of ALPS. Virtually all patients have experienced protracted periods of palpable, nontender lymph node enlargement, but the dimensions of the nodes can fluctuate, tending to become relatively less impressive through adolescence and adulthood. On the other hand, lymphadenopathy may be massive, distorting anatomical landmarks, as seen in the 5-year-old child depicted in Plate 30.I, in whom the lymphoid mass varied but did not abate through 10 years of serial clinical observations. The anterior, posterior cervical, and axillary chains in this patient were the primary sites of enlarged lymph nodes, but, as seen in the rear view, there was also enlargement of posterior nodes. Some patients have had enlargement of preauricular and submental nodes as well as nodes in the axillary, epitrochlear, and inguinal chains. Mediastinal and retroperitoneal adenopathy can be detected on imaging studies (Avila et al., 1999). Canale and Smith (1967) observed that intercurrent infections were associated with reductions in lymph node size, and patients followed at NIH have been observed to have similar paradoxical decreases in lymph nodes in a few, but not most, instances of documented infections.

AUTOIMMUNITY

Autoimmunity in ALPS is largely directed against blood cells. Direct Coombs-positive autoimmune hemolytic anemia (AIHA) is most common autoimmune complication (Table 30.4), immune-mediated thrombocytopenia (ITP) second, while autoimmune neutropenia has been proven less frequently, possibly due to a lack of sensitive standardized tests for antineutrophil antibodies. Episodes of hemolysis may be severe; many patients have experienced at least one occasion on which hemoglobin levels fell below 7 mg/dL. Similarly, platelet counts below 10,000/μL have been seen one or more times in almost half of the patients followed at NIH (Sneller et al., 1997).

In a small number of ALPS patients, glomerulonephritis with renal compromise has been severe enough to require dialysis, and Guillain-Barré polyneuroradiculitis has occurred without any recognized predisposing infectious illness (Rieux-Laucat et al., 2003; Sneller et al., 1997). Many ALPS patients suffer from recurring rashes, including urticaria and nonspecific cutaneous vasculitis. Other infrequently reported findings of unclear pathogenesis in ALPS patients have included arthralgia, arthritis, hepatitis, biliary cirrhosis, iridocyclitis or uveitis, mucosal ulcers, panniculitis, pulmonary infiltrates, seizures, and vasculitis (Rieux-Laucat et al., 2003; Sneller et al., 2003).

Autoimmunity is a hallmark in ALPS, but it can be difficult to document for three reasons. First, not all patients are tested for many recognized types of autoantibodies. Second, some patients manifest overt autoimmune phenomena only after many years of lymph node and spleen enlargement. For example, one patient seen at NIH first developed ITP at age 31, while another first manifested AIHA at age 54. Finally, in the absence of demonstrable antiplatelet, antineutrophil, or anti-red cell antibodies, one cannot diagnose with certainty an immune basis for blood cytopenias in a setting of ongoing hypersplenism. Additional patients such as those in the reports of Le Deist et al. (1996) and Bettinardi et al. (1997) who were recognized because of their more severely affected siblings are so far without overt autoimmune disease but do have circulating autoantibodies.

MALIGNANCY

In some patients with ALPS a diagnosis of lymphoreticular malignancy or premalignant state was made but later reversed upon review of the histology in lymph node biopsies. However, in one of the earliest reports of ALPS, Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) were confirmed in two young adult brothers with ALPS, one of whom died of this complication (Fisher et al., 1995). An unrelated ALPS patient treated for confirmed NHL had an affected cousin who developed HD at age 7, and another unrelated woman and her son with ALPS had HD (Infante et al., 1998). A multicenter study determined that in patients with ALPS-FAS (formerly ALPS type Ia) the risk of NHL was 14-fold and the risk of HD 51-fold above that of the general population (Straus et al., 2001). Lymphomas reported to date have been exclusively B-cell–derived and included various subtypes of HD, Burkitt's lymphoma, follicular B-cell lymphoma, and T-cell–rich B-cell lymphoma. The overall rate of lymphoma among ALPS-FAS patients followed at NIH has been 9 percent as of this writing (Table 30.4; Rao et al., 2010; Sneller et al., 2003). Lymphoma has been diagnosed in patients up to 51 years of age. As young patients are followed for increasing durations of time, the overall rate of lymphoma may increase.

Two older ALPS patients reported by Drappa et al. (1996) had malignancy. One, who died of hepatocellular carcinoma at age 43, had risk factors including hepatitis C and a history of prior cytotoxic chemotherapy for his adenopathy. The other had multiple thyroid and breast adenomas and two basal cell carcinomas, all occurring between the ages of 15 and 41. In addition, another ALPS patient developed a histiocytic sarcoma (Rao et al., 2010). Other solid organ malignancies have occurred in some of the older relatives of ALPS patients, but neither their frequency nor any distinctive features recognized to date clearly distinguish them from malignancies in the general population.

OVERLAP BETWEEN ALPS AND OTHER SYNDROMES

With increased awareness in the medical community, patients previously diagnosed with other conditions have been recognized to have ALPS. Evans syndrome is a hematological disorder of unknown etiology consisting of multiple autoimmune blood cytopenias, most commonly hemolytic anemia and thrombocytopenia. Enlargement of lymph nodes, spleen, and liver occur in over half of the cases. First described by Evans et al. (1951), this syndrome is clinically similar to ALPS. An initial examination of 12 patients with Evans syndrome identified ALPS in half of them (Teachey et al., 2005), and a multi-institutional study of 45 children with Evans syndrome confirmed 22 with a diagnosis of ALPS (Seif et al., 2010). These studies suggest consideration

of a diagnosis of ALPS and with initial screening by enumeration of αβ DNT cells in all cases of Evans syndrome.

Children with sinus histiocytosis with massive lymphadenopathy (SHML), also known as Rosai-Dorfman disease, share some clinical features with ALPS, including prominent adenopathy, hypergammaglobulinemia, and autoimmune phenomena in 10 to 15 percent of cases (Grabczynska et al., 2001; Rosai and Dorfman, 1969, 1972). Histological examination of tissues from 44 patients with confirmed ALPS-FAS (at the time categorized as ALPS Ia) revealed in 18 cases the characteristic SHML histiocytic proliferation with emperipolesis (enlarged histiocytes that have engulfed other blood cells) and prominent expression of the S-100 antigen that typifies Rosai-Dorfman syndrome (Maric et al., 2005). A preliminary report has also noted *FAS* mutations in DNA isolated from tissues of SHML patients (George et al., 2004).

Although the original mouse with Fas deficiency was developed as a model for human systemic lupus erythematosus (SLE), *FAS* mutations have not been found commonly in patients with SLE. Nonetheless, at least one patient each with ALPS-FAS, ALPS-FASL, and ALPS-CASP10 confirmed by mutation analysis has fulfilled the diagnostic criteria for SLE (Gill et al., 2003; Vaishnaw et al., 1999a; Wu et al., 1996; Zhu et al., 2006).

Finally, the recognition of increased risk of lymphoma in ALPS-FAS patients suggests that with other forms of ALPS, inherited mutations in the receptor-mediated apoptosis pathway may underlie a proportion of sporadic occurrences of this malignancy, and possibly a considerable proportion of familial lymphomas. Somatic mutations in *FAS* are already known to occur in some 10 percent of cases of B-cell lymphomas and leukemias. The demonstration of somatic mutations in ALPS (Dowdell et al., 2010; Holzelova et al., 2004) indicates that clinical features of autoimmunity and lymphoproliferation may long predate the actual emergence of clonal lymphoproliferation.

PATHOLOGICAL FEATURES OF ALPS

Histopathological analysis of lymph nodes of patients affected with ALPS shows characteristic pathological changes (Lim et al., 1998; Sneller et al., 1992), as seen in Plate 30.II. There is architectural preservation, but with a florid reactive follicular hyperplasia with immunoblasts and plasma cells and marked paracortical expansion. The features resemble those of viral lymphadenitis except for the conspicuous absence of histiocytes normally seen to contain apoptotic debris in infection-associated reactive nodes. The paracortical expansion is in some cases extensive enough to consider a differential diagnosis of immunoblastic lymphoma, with many cells expressing the Ki-67 antigen, indicative of active proliferation (Gerdes et al., 1984).

Analysis of splenic tissue from ALPS patients demonstrates lymphoid hyperplasia of the white pulp with histological features similar to those of the lymph nodes. B cells expand the lymphoid follicles while DNT cells accumulate in the paracortical areas. Bone marrow aspirates are generally unremarkable, although trilineage hematopoiesis is seen in patients with autoimmune cytopenias.

Although many of the T cells in both spleen and lymph nodes are $CD4^+$ or $CD8^+$, the remarkable and most characteristic feature of the lymphoid histology in ALPS is the large proportion of α/β TCR $CD4^-$ $CD8^-$ cells in the paracortical areas. Given the underlying defects in apoptosis in ALPS, it was surprising when histological analyses showed many splenic lymphocytes undergoing apoptosis (Le Deist et al., 1996). It is presumed that alternative pathways of lymphocyte apoptosis may be upregulated in an attempt to compensate for a defect in the FAS-mediated apoptosis pathway. As discussed above, histiocytic infiltrates and emperipolesis of lymphocytes and plasma cells, as seen in SHML, are found in biopsied lymph nodes of about 40 percent of ALPS cases. The enlarged histiocytes, located in paracortical areas and sinusoids, are strongly positive for the S-100 antigen (Maric et al., 2005).

In ALPS-CASP10, impairment of dendritic cell apoptosis was noted and associated in one patient with expansion of dendritic cells in lymph node biopsy (Wang et al., 1999).

LABORATORY FINDINGS IN ALPS

IMMUNOLOGICAL FINDINGS

Lymphocyte phenotyping provided the first clues as to the unique nature of ALPS. There is an increase of peripheral blood $CD3^+$ T cells that exceeds the sum of $CD4^+$ plus $CD8^+$ cells, indicating expansion of a normally minor $CD4^-$ $CD8^-$ T-cell or DNT-cell subset. Such cells can be found in the blood of some patients with malignancy, HIV infection, acute viral infections, and certain other inflammatory states. However, in these disorders, the expanded cell subpopulation typically bears γ/δ TCR. In contrast, the DNT-cell subpopulation that is increased in all patients with ALPS expresses the α/β TCR. This population is less than 2.5 percent of $CD3^+$ T cells (1.5 percent of lymphocytes) in normal controls and over 200 FAS-normal relatives of ALPS patients, while ALPS patients typically have 5 to 20 percent DNT cells, with a range from 1.5 to 68 percent (Bleesing et al., 2001; Oliveira et al., 2010). These cells are also generally $CD45RA^+$, $CD45RO^-$, $CD57^+$, and many of them have increased expression of HLA class II antigen, HLA-DR (Bleesing et al., 2001; Sneller et al., 1997). The DNT cells in ALPS express restricted V-beta TCR diversity and appear to be clonally related to $CD8^+$ T cells (Bristeau-Leprince et al., 2008). As distinct from $CD4^-$ $CD8^-$ immature thymocytes, the DNT cells of ALPS, when isolated and studied in vitro, are poorly responsive to mitogens and antigens and fail to produce cytokines such as IL-2 upon activation (Sneller et al., 1992). Rather than immature cells, they appear to be a population of aged T cells that have escaped elimination by apoptosis. They thus are a marker for the ALPS disease state, but their role in the evolution of autoimmunity remains unknown.

Patients with ALPS also show other abnormalities in their immunological profiles (Bleesing et al., 2001, 2002; Table 30.5). In addition to $CD4^+$ and $CD8^+$ T cells displaying

Table 30.5 LABORATORY FINDINGS IN ALPS

IMMUNOLOGY
Lymphocytes
Relative if not absolute lymphocytosis involving both B and T cells
Excess of $CD4^-/CD8^-$ T cells; specifically >1.5% α/β TCR $CD4^-/CD8^-$ cells
Increased proportions of HLA DR^+ and $CD57^+$ T cells
Decreased $CD4^+/CD25^+$ T cells
Decreased CD27 expression on B cells (with increased soluble CD27 in serum)
Decreased delayed-type hypersensitivity to skin test antigens
Granulocytes
Neutropenia
Eosinophilia
Immunoglobulins
Elevated levels of IgG, IgA, and/or IgM
Monoclonal IgG1 spike in one severely affected patient with homozygous Fas deficiency
Poor and/or unsustained specific antibody responses to polysaccharide antigens
Autoantibodies to
Erythrocytes (direct Coombs)
Platelets
Neutrophils
Phospholipids
Smooth muscle
Rheumatoid factor
Antinuclear
Cytokines
Elevated serum IL-10 (ALPS-FAS and ALPS-sFAS)
Elevated serum IL-18 (ALPS-FAS and ALPS-sFAS)
Elevated sFASL (ALPS-FAS and ALPS-sFAS)
Hematology
Anemia
Hypersplenism
Autoimmune hemolysis
Iron deficiency
Elevated vitamin B12 levels
Chemistry
Elevated aminotransferases or cholesterol seen uncommonly
Proteinuria (in cases of glomerulonephritis)

HLA-DR and CD57, ALPS patients average a 5-fold or greater expansion of B-cell numbers relative to normal subjects, and the B cells have a marked decrease in the expression of the memory marker CD27 (Bleesing and Fleisher, unpublished). Natural killer (NK) cell numbers are normal. ALPS patients have a characteristic TH2 T-helper-cell–oriented cytokine profile, with increased TH2 cytokines IL-4, IL-5, and IL-10 and reduced in vitro release of the TH1 cytokines IL-12, IL-2, and interferon-γ. Levels of IL-10 in serum are profoundly elevated in most patients with ALPS, particularly patients with *FAS* mutations, whereas they are usually undetectable in normal subjects and patients without ALPS, even those who have a variety of autoimmune disorders characterized by autoantibodies, such as SLE (Fuss et al., 1997). The TH2 cytokine profile is thought to promote the development of autoimmune features of ALPS.

Recently a pattern of biomarkers has been identified that correlates with a diagnosis of ALPS-FAS. These include marked elevations in serum IL-10, IL-18, and soluble FASL levels as well as increased serum vitamin B12 levels (Caminha et al., 2010; Magerus-Chatinet et al. 2009).

Despite a dramatic and at times alarming degree of lymphoid organ enlargement, immunity in ALPS is surprisingly intact unless disease complications are being treated with immunosuppressive medications. Except for frequent pneumococcal sepsis following splenectomy, ALPS-FAS patients have rarely experienced unusual or severe opportunistic infections. Nonetheless, abnormalities in both humoral and cellular immunity can be demonstrated. Of eight NIH patients tested, four failed to respond to a panel of three delayed-type hypersensitivity skin test recall antigens. Although antibody responses to T-dependent antigens appeared to be intact, patients with ALPS responded less to polysaccharide antigens, such as those in the 23 serotype pneumococcal polysaccharide vaccine. Moreover, several patients have failed to sustain initially protective antibody levels to pneumococcal polysaccharides following immunization, a concern in asplenic individuals who are susceptible to bacterial sepsis. Antibodies to the T-dependent antigen tetanus toxoid have also been noted to be poorly sustained. Immunoglobulin levels are usually elevated, with IgG concentrations as high as 8 g/dL. IgA or other isotypes may be elevated. The immunoglobulin is almost invariably polyclonal, but Rieux-Laucat et al. (1995) reported one child with a monoclonal IgG-1 spike.

Autoantibody production is very common in ALPS, with most antibodies directed against red cells or platelets. Antineutrophil antibodies and low titers of anti-smooth muscle, antiphospholipid, antinuclear antibodies, and rheumatoid factor can also be seen.

HEMATOLOGY

Most ALPS patients are anemic based on one or more of three mechanisms: hypersplenism, Coombs-positive hemolysis, and iron deficiency. Prior to splenectomy it is not uncommon for hemoglobin levels to average 7 to 8 mg/dL. Hemoglobin concentrations below 3 mg/dL can be seen in acute hemolytic crises. Red cell indices are mostly normocytic, but microcytosis is common, and red cell survival is short.

Platelet counts can be normal, but in the presence of hypersplenism they are typically low, and they are elevated

after splenectomy. Thrombocytopenic crises with bruising and epistaxis are frequent immune-mediated events.

Nearly all patients with ALPS sustain an absolute lymphocytosis ranging from 8,000 to 90,000 cells/μL or even higher, but averaging about 14,000 cells/μL after splenectomy. Granulocytosis can occur. On the other hand, an absolute neutropenia is common prior to splenectomy. Postsplenectomy immune-mediated neutropenia is well documented (Kwon et al., 2003). Most patients also exhibit a relative, if not absolute, eosinophilia ranging from 3 to 32 percent and averaging about 7 percent a finding that has been associated with abnormal Fas expression in a murine model (Simon et al., 1996).

CHEMISTRY

The blood chemistry profile in ALPS is largely normal, but occasional patients have persistently elevated aminotransferases in the range of 100 to 300 IU/mL. One individual in the NIH series had hypercholesterolemia associated with lymphocytic infiltration into the hepatic sinusoids and portal triads; liver transplantation was eventually required (S. E. Straus, unpublished). While this patient did not have any evidence of viral hepatitis, a patient with ALPS and hepatitis C developed hepatocellular carcinoma (Drappa et al., 1996).

MOLECULAR BASIS OF ALPS

The similarity between humans with ALPS and mice with *lpr* and *gld* mutations was postulated in 1992 in a report from NIH of two children with massive, nonmalignant lymphoid hyperplasia, autoimmune disease, and markedly elevated numbers of $CD4^-$ $CD8^-$ T cells (Sneller et al., 1992). The *lpr* mouse had been studied for many years as a model for immune complex diseases, particularly lupus, and was known to have lymphoid hyperplasia and expanded $CD4^-$ $CD8^-$ T cells as well as nephritis due to renal deposition of autoantibodies (Cohen and Eisenberg, 1991; Theofilopoulos et al., 1981). Shortly after the NIH patients were reported in 1992, the genetic cause of the murine *lpr* phenotype was discovered to be a homozygous autosomal recessive mutation causing extremely reduced levels of the protein designated Fas (for *F*S-7 cell line-associated surface antigen) (Watanabe-Fukunaga et al., 1992; Yonehara et al., 1989). The same protein was independently identified and named APO-1 (Dhein et al., 1992; Oehm et al., 1992; Trauth et al., 1989), and a third name, CD95, was subsequently assigned.

In both the Japanese group led by S. Yonehara and S. Nagata and the German group led by P. H. Krammer, monoclonal antibodies had been developed that induced apoptosis upon binding to the surface of cells (and the FS-7 cell line in particular). In addition to their effect of cross-linking a cell-surface apoptosis receptor, these antibodies were used to purify the FAS/APO-1/CD95 protein, which in turn was partially sequenced, leading to identification and isolation of the human cDNA for the apoptosis gene now officially designated *FAS* (*TNFSF6*, *CD95*, *APT1*). Furthermore, the same antibodies were used to demonstrate the absence of Fas on lymphocytes of the *lpr* mouse, indicating that defective lymphocyte apoptosis was the basis of the autoimmune phenotype of this mouse. In related studies, the defect in *gld* mice proved to be in the Fas ligand (FasL), which cross-links Fas under physiological conditions to induce apoptosis (Fig. 30.1; Takahashi et al., 1994). Both Rieux-Laucat et al. (1995) and Fisher et al. (1995) described defects in the human *FAS* gene in patients with autoimmunity, lymphoproliferation, and excess $CD4^-$ $CD8^-$ T cells, including one of the original NIH patients described by Sneller et al. (1992).

Fas and FasL are members of two superfamilies of receptors and ligands that are important in immune regulation (reviewed in Lenardo et al., 1999; Nagata and Goldstein, 1995). Fas is a member of the tumor necrosis factor receptor superfamily (TNFRSF), which includes TNFRSF1A (p55) and TNFRSF1B (p75); CD40, important in B-cell activation (see Chapter 26); CD30, found on Reed-Sternberg cells in Hodgkin's lymphoma; and several other receptors. These type 1 membrane-spanning proteins share up to 25 percent amino acid identity and contain variable numbers of conserved extracellular cysteine-rich domains (CRDs). Fas and TNFRSF1A share an intracellular region of homology as well, a 70 amino acid "death domain," around which is assembled a death-inducing signal complex (DISC) that propagates intracellular signals for apoptosis.

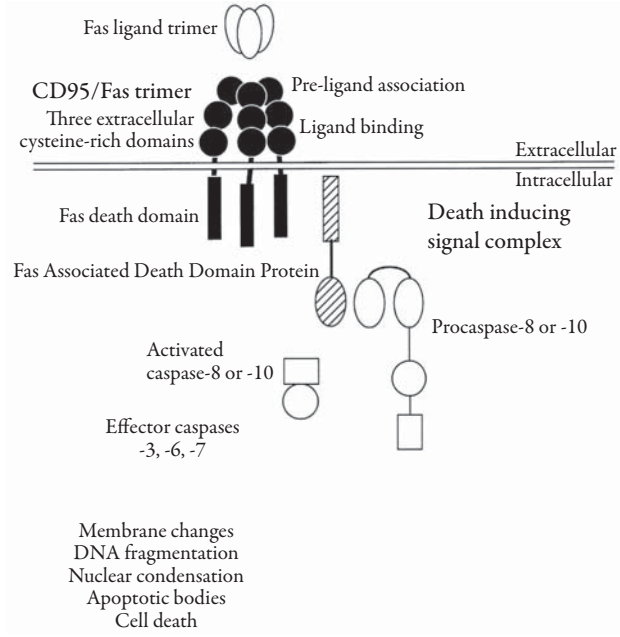

Figure 30.1 Lymphocyte apoptosis pathways (Nagata et al., 1997). After Fas, TNFR-1, and related apoptosis mediators (not shown) are linked by their trimeric ligands, their intracellular death domains transduce signals to cytoplasmic death domain-containing proteins, including FADD/MORT1, TRADD, and RIP. Caspase protease domains of FADD/MORT1 and FLICE/MACH1 cleave downstream proteases such as caspase 8, which in turn signal the activation of proteolytic enzymes and DNases. Some components of the apoptosis pathway such as RIP and TRAF-2 may have a regulatory role, inducing antiapoptotic survival factors via NF-κB activation.

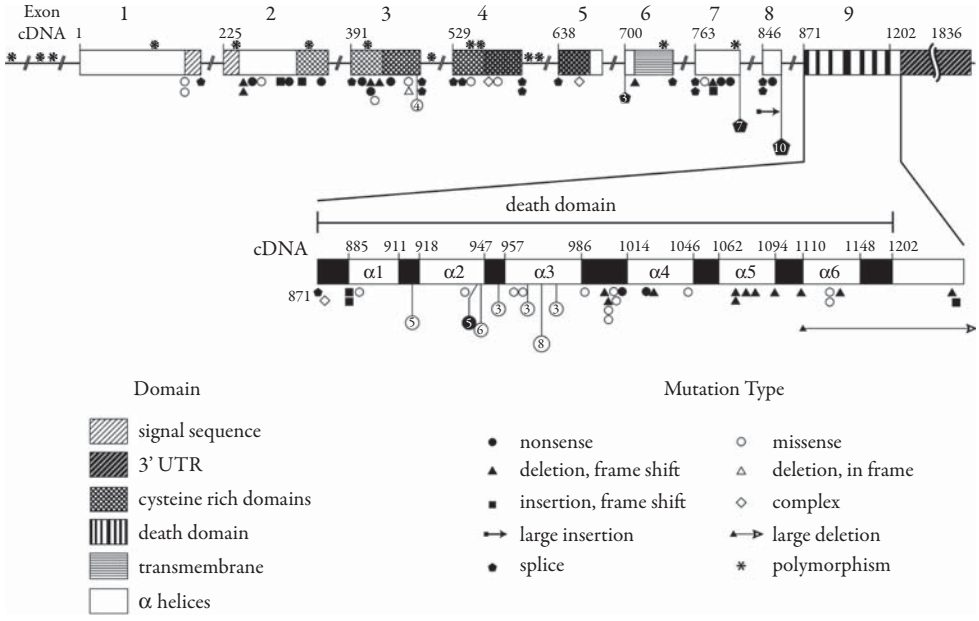

Figure 30.2 Diagram of the structure of the human *FAS* gene encoding the protein FAS/CD95/APO-1, showing mutations in patients with ALPS. CRD, cysteine-rich domain. (A) Two heterozygous alleles bearing missense mutations in three brothers with ALPS reported in Italy (Bettinardi et al., 1997). (B) Homozygous deletion resulting in undetectable cell surface Fas protein in a severely affected patient reported from France (Le Deist et al., 1996; Rieux-Laucat et al., 1995). Mutations in the top row are reported from Europe, those in the second row are from Cornell University Medical Center (Drappa et al., 1996), and the remaining mutations were detected at NIH (Fisher et al., 1995; Infante et al., 1998; Sneller et al., 1997; NIH group, unpublished). GenBank accession numbers X81335–X81342 (Behrman et al., 1994).

Protein structural studies indicate that FASL, which occurs in membrane-bound and secreted forms, self-associates into homotrimers with a conical configuration to become functionally active (Fig. 30.1). The FASL trimer engages a homotrimeric complex of Fas chains displayed on the surface of lymphocytes. FAS chains self-assemble at the cell surface by means of their pre-ligand association domains in the first CRD of the FAS molecules (Siegel et al., 2000). Upon ligand engagement, the extracellular projections of the Fas trimeric complex extend and embrace the FasL trimer. Disulfide bonds between the cysteine residues of the CRDs stabilize the conformation of the extracellular portion of Fas.

Extracellular binding of a FASL trimer to a FAS trimer results in the formation at the intracellular side of the cell membrane a DISC that consists of the trimeric FAS death domains, the Fas-associated death domain protein FADD (also called MORT1), TRADD, RIP, and associated partners that possess proteolytic ICE-like domains (named for their homology to IL-1β converting enzyme) (Enari et al., 1995; Hsu et al., 1995, 1996; Los et al., 1995). These ICE-like proteases, now collectively referred to as *caspases*, cleave proteins at specific amino acid recognition sites. Procaspase 8 (originally called FLICE and MACH by different independent groups of discoverers) and procaspase 10 can be cleaved by the proteolytic domain of FADD/MORT1. After cleavage, caspase 8 and caspase 10 themselves becomes active proteases that act on further proteases in a cascade, eventually leading to activation of effectors caspase 3 and caspase 9, which in turn bring about the cellular events that constitute the apoptosis program (Salmena et al, 2003). Characteristic lethal cellular events collectively known as apoptosis include early membrane permeabilization and exposure of proteins not normally detectable at the cell surface; DNA cleavage between nucleosome units into fragments of incremental sizes, visible on electrophoresis gels as "ladders;" condensation and segmentation of nuclei; and shedding of "apoptotic bodies," nuclear particles encased in membrane that are rapidly phagocytosed by macrophages (Lenardo, 1996; Nagata and Goldstein, 1995). Apoptosis, in contrast to necrosis, the other major physiological mechanism for removal of cells in the body, does not incite local inflammation.

Figure 30.2 shows the organization of the *FAS* gene encoding FAS; the gene has nine exons within a genomic span of about 25 kb on human chromosome 10q23 (Behrmann et al., 1994). The open reading frame, beginning in exon 2, encodes a signal peptide of 16 amino acids. After directing transmembrane expression this peptide is cleaved off the mature protein. Exons 2 through 5 encode the three extracellular CRD units of FAS; the sequences encoding the transmembrane domain lie within exon 6. The intracellular domain of *FAS* includes in exon 9 a death domain with homology to the intracellular portion of *TNFRSF1A*. A number of single nucleotide polymorphisms (SNPs) that do not alter amino acid sequence have been defined within the *FAS* gene (Neimela et al., 2006). Particularly common SNPs in exon 3 and exon 7 (Fiucci and Ruberti, 1994) make possible allotype assignment for family genetic studies. The FAS protein is expressed in heart and liver as well as in lymphocytes. Particularly high amounts are found in T cells activated by TCR engagement and IL-2.

MUTATION ANALYSIS AND GENOTYPE–PHENOTYPE CORRELATIONS IN ALPS-FAS

To date, over 500 patients with ALPS have been identified (Jackson et al., 1999; Kasahara et al., 1998; Le Deist et al., 2003; Oliveira et al., 2010; Puck, 2005; Vaishnaw et al., 1999a, 1999b). Among these, *FAS* gene mutations causing ALPS-FAS have been found in over 200 families, and several families have more than one affected member (Bettinardi et al., 1997; Drappa et al., 1996; Infante et al., 1998; Le Deist et al., 1996; Oliveira et al., 2010; Puck, 2005; Sneller et al., 1997). A website that lists many curated mutations in *FAS* is the Leiden Open Variation (LOVD) Database installation at the National Center for Biotechnology (NCBI) at NIH (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db = FAS). The above mutations, other than the polymorphisms mentioned above and other SNPs that do not alter the predicted amino acid sequence of the FAS protein, have not been detected upon screening over 200 alleles of chromosome 10 from unrelated healthy individuals.

Nearly all of the mutations causing ALPS-FAS to date are changes of a single nucleotide or a few nucleotides in *FAS*. Exceptions are a 331-bp insertion 5′ of exon 8 that disrupts splicing and a 290-bp terminal deletion in exon 9 (Fig. 30.2). The latter deletion was found in homozygosity in a very severely affected daughter of consanguineous parents (Le Deist et al., 1996; Rieux-Laucat et al., 1995). The infant had massive lymphoproliferation at birth, evidenced by hydrops fetalis with massive hepatosplenomegaly. The spleen had to be removed at the age of 2 months, at which time it weighed 1.8 kg. Other findings were pulmonary infiltrates, intra-abdominal lymph node enlargement as shown by CT scan examination, and lymphocytosis up to 150,000/μL. Although a *FAS* mRNA signal of reduced length was detected by Northern analysis in this patient, consistent with deletion of the 3′ end of the gene, FAS protein was not detectable by cell-surface staining with anti-FAS antibody, suggesting a defect either in protein synthesis or in stability or transport to the cell membrane. The heterozygous parents of this patient were healthy, and their lymphocytes had normal FAS-mediated apoptosis by the in vitro testing method employed (Le Deist et al., 1996). Thus, this human null mutation was most reminiscent of the *lpr* mouse mutation in that it resulted in nonexpression of FAS and a recessive, severe disease phenotype.

The three brothers with striking lymphoproliferation characteristic of ALPS reported by Bettinardi et al. (1997) were compound heterozygotes for two missense mutations, one in the extracellular CRD2 domain and the other at the proximal end of the intracellular death domain. One brother had autoimmune thrombocytopenia and hemolytic anemia, while the others had no overt autoimmune disease but did have autoantibodies. Interestingly, their parents, each a heterozygous carrier with one wild-type *FAS* allele, were reported to be healthy and without elevated DNT cells or defective apoptosis. The mechanism by which the two mutations in these brothers might combine to produce ALPS with recessive phenotype is not clear.

By far the majority of ALPS-FAS cases are associated with heterozygous *FAS* mutations; ALPS is inherited in an autosomal dominant fashion along with FAS defects in most kindreds. Patients with this form of ALPS have one mutant and one normal, functional *FAS* allele. As shown in Figure 30.2, the most common region of the *FAS* gene to be mutated in ALPS-FAS is the intracellular death domain in exon 9, expanded in the figure to show the large number of mutations clustered within it. The heterozygous death domain mutations occur in and around the six α-helical regions of the peptide structure (Huang et al., 1996). These mutations are predicted to result in either early termination of protein synthesis (frameshifting insertions and deletions; amino acids changed to stop codons) or single amino acid substitutions (missense mutations), as shown in Figure 30.2. They occur in portions of the death domain that are highly conserved in other death domain proteins in other species such as the mouse. For example, a point mutation in murine Fas in the lpr^{cg} mouse strain has been identified and found to predispose to lymphoproliferation and autoimmune disease similar to that in mice with the *lpr* and *gld* mutations (Kimura and Matsuzawa, 1994; Watanabe-Fukunaga et al., 1992). This missense point mutation in the lpr^{cg} mouse introduces a nonconserved asparagine residue corresponding to human FAS amino acid 238, between the second and third α-helices. Immediately adjacent to the position of this mouse mutation, three human missense mutations, G237V, G237S, and G237D, have been identified in patients with ALPS. The heterozygous death domain substitution mutations are compatible with expression of mRNA and protein, suggesting that mutant protein molecules may inhibit apoptosis by normal FAS expressed concurrently. Ultrastructural studies have indicated disruption of the death domain architecture by some mutations, whereas others appear to affect contact points between FAS and FADD (Huang et al., 1996).

Many other mutations in the extracellular domains of *FAS* as well as in the more proximal intracellular regions result in clinical and immunological presentations similar to those of ALPS patients with death domain mutations, but often of milder clinical severity. As an example, one patient with a splice mutation immediately following exon 3 was shown to make in-frame abnormal mRNA and protein that skipped exon 3 (Fisher et al., 1995; this individual is pictured in Plate 30.I). Two other patients had mutations within exon 3, a nonsense mutation and a single base deletion with a frameshift. Curiously, unlike the splice mutation described above, analysis of cDNA clones showed that these mutant alleles were not associated with exon 3 skipping (Jackson et al., 1999; Puck, unpublished). If translated, they would produce truncated proteins corresponding only to the first CRD. Splice mutations of exons encoding intracellular domains have also been found, in some cases producing a shortened mRNA and protein terminating immediately after the transmembrane region, but without intracellular charged amino acids to anchor it (Fisher et al., 1995). The possibility that some mutations encode shortened FAS proteins that are secreted from the cell needs to be explored. These mutations may cause disease through the mechanism of haploinsufficiency (Hsu

et al, 2012; Kuehn et al, 2011). Expression and function of mutant alleles must be addressed experimentally to determine whether and by what mechanism it is deleterious.

Satisfactory correlations between the *FAS* genotype and the clinical presentation or phenotype of each individual are complicated by several factors. First, as indicated above, ALPS may be recessive, but it is more often a dominant disorder associated with heterozygous mutations; the mode of inheritance is at least in part dependent on the type of mutation itself, with homozygous or compound heterozygous mutations expected to produce more severe, recessive disease. Second, penetrance and expressivity of phenotypes associated with heterozygous *FAS* mutations are also variable, in that mutation-bearing relatives of ALPS probands may have all of the defining criteria for ALPS or may instead have milder or even completely undetectable clinical and immunological abnormalities (Infante et al., 1998; Jackson et al., 1999; Vaishnaw et al., 1999b).

FUNCTIONAL ASPECTS: IN VITRO ASSAY OF APOPTOSIS

Defective apoptosis is the hallmark of peripheral blood lymphocytes of patients with ALPS. Activated, cultured T cells from ALPS patients exhibit reduced rates of apoptotic death when stimulated through the FAS pathway (Fig. 30.3A). To assay apoptosis, peripheral blood mononuclear cells in vitro are initially exposed to a strong activating stimulus such as phytohemagglutinin; then the activated T cells from these cultures are maintained and expanded in IL-2; and after 5 to 20 days, the cells are subjected to trimerized FAS ligand or an anti-FAS monoclonal antibody, such as CH11 or Apo-1 (Fisher et al., 1995). Apoptosis can be quantitated by a variety of means, perhaps most directly by measuring loss of viable cells in culture wells exposed to antibody versus no antibody for 24 hours. As shown in Figure 30.3A, after cross-linking activated cultures of lymphocytes from four ALPS patients exhibited rates of cell loss averaging only 7 percent, compared to an average of 50 percent for cultures from three healthy control subjects. Other methods of demonstrating apoptosis include staining free DNA ends that result from DNA fragmentation and demonstrating characteristic membrane changes. Recently an alternative assay called the FasT Kill Assay that is performed on fresh PBMCs and yields reproducible results within hours (Lo et al, 2013). Assay conditions and normal ranges for all apoptosis tests must be established and monitored in each laboratory.

Lymphocytes from all ALPS patients with deleterious germline *FAS* mutations have defective FAS-specific apoptosis. ALPS patient B-cell lines transformed with Epstein-Barr virus likewise show defective apoptosis after anti-FAS exposure, indicating that both B and T lymphocytes express the defect in FAS-mediated programmed cell death (Sneller et al., 1997). In addition, mutation-bearing family members, even those with no clinical abnormalities and no elevations of $CD4^-$ $CD8^-$ T cells, demonstrate defective apoptosis in vitro. Thus the impairment in cellular apoptosis associated with *FAS* mutation is inherited as a dominant trait.

The NIH group (Fisher et al., 1995) studied the mechanism by which a heterozygous mutant *FAS* allele can exert a dominant inhibition of apoptosis by using an in vitro transfection system (Fig. 30.3B). *FAS* cDNA in an expression plasmid introduced into a FAS-negative mouse thymoma cell line by electroporation achieved measurable cell-surface expression of mutant or normal FAS in about 50 percent of the target cells (Fig. 30.3B, white bars). When the human-specific CH11 anti-FAS monoclonal antibody was added (black bars), 50 percent of cells treated with 10 μg of normal *FAS* plasmid were killed; thus virtually all of the FAS-expressing cells died. When thymoma cells were transfected with *FAS* cDNA bearing the death domain mutation T225P found in an ALPS patient, protein was expressed at the cell surface, but there was no apoptotic cell loss after treatment with CH11. As shown on the right of the figure, when mixtures of normal and mutant *FAS* cDNA were transfected together, the mutant allele inhibited transmission of a death signal by the normal, wild type allele (Fig. 30.3, asterisks). With equal amounts of wild-type and mutated *FAS* cDNA, apoptosis was almost completely interrupted; this dominant inhibitory effect was still seen with a 3:1 ratio of wild type to mutant.

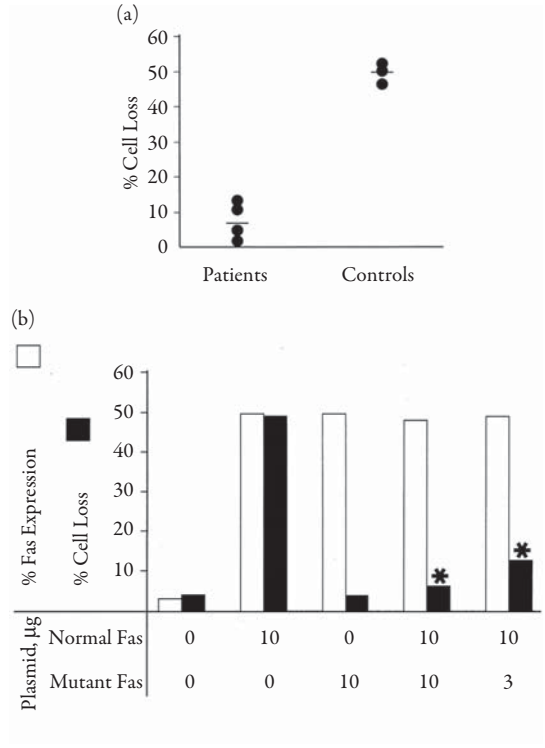

Figure 30.3 FAS-mediated apoptosis. (A) FAS-mediated killing of activated, IL-2–dependent T cells derived from ALPS patients versus normal controls. The percent cell loss reflects the difference between cells exposed to an anti-FAS monoclonal antibody compared to cells not exposed to the antibody (Fisher et al., 1995). (B) Expression (*open bars*) and apoptosis (*black bars*) in cells transfected in vitro with *FAS* cDNA constructs, followed by exposure to anti-FAS antibody to induce cell loss. Transfection with normal *FAS* leads to killing of all cells expressing FAS, while essentially no cell loss occurs with mutant FAS. Transfection with mixtures of normal and mutant *FAS* show the dominant negative inhibition of apoptosis by mutated FAS (*) despite the presence of normal FAS.

The dominant-interfering effect on apoptosis of FAS proteins with a death domain mutation is consistent with the model of FAS as a functional trimer (Fisher et al., 1995). Three FAS molecules form a trimeric complex to interact with a FASL trimer at the cell surface, and the intracellular death domains of the FAS molecules must interact properly with downstream mediators in the DISC to accomplish cell killing. If there is equal expression of normal and mutant FAS mRNA, normal and defective protein chains are expected to be synthesized and expressed equally at the cell surface. Assembly of these FAS monomer chains into trimers at random will yield only one out of every eight trimer complexes with three normal FAS subunits. Following this model, the presence of one, two, or three defective FAS molecules in a complex is predicted to render that complex nonfunctional. Thus, heterozygous interfering mutations are expected to have a profound inhibitory effect on apoptosis, as is actually observed.

Some FAS proteins with mutations outside of the death domain may also have a dominant inhibitory effect (Fisher et al., 1995; Jackson et al., 1999). Similarly, truncated FAS protein molecules terminating before or within the death domain may impair apoptosis in a dominant negative fashion (Jackson et al., 1999; Rieux-Laucat et al., 1999; Vaishnaw et al., 1999b). On the other hand, certain mutations, and indeed some splice variants of normal FAS, produce a protein that lacks the transmembrane region and thus may be secreted rather than membrane-bound (Cheng et al., 1994; Papoff et al., 1996; Ruberti et al., 1996). In vitro studies by some investigators indicate that peptides containing the first 49 amino acids of FAS are sufficient to inhibit apoptosis in cultured cells (Papoff et al., 1996). However, the pathophysiological role of secreted FAS in ALPS or in other autoimmune disorders, whether produced by mutant alleles or splice variants, remains unclear. Furthermore, some *FAS* mutations, such as termination codons at the far 5′ end of the gene are null, resulting in loss of function rather than dominant interference (Jackson et al., 1999; Vaishnaw et al., 1999b). These mutations appear to cause ALPS by producing haploinsufficiency, implying that the amount of FAS at the cell surface is a critical parameter for induction of apoptosis in physiological conditions (Hsu et al., 2012; Kuehn et al., 2011).

ALPS DUE TO SOMATIC MUTATIONS OF FAS

Holzelova et al. (2004) initially reported a group of six children presenting by 2 years of age with typical clinical features of ALPS, including lymphadenopathy, splenomegaly, elevated DNT cells, hypergammaglobulinemia, and, in four of six cases, autoimmune disease. Interestingly, none of the patients had affected relatives; moreover, they lacked demonstrable defects in lymphocyte FAS-mediated apoptosis and did not have germline mutations of *FAS*. The DNT cells of these patients were isolated by flow cytometric sorting, and DNA prepared from this cell subset was subjected to *FAS* sequence analysis. In all six DNT samples, deleterious *FAS* mutations either identical to or having the same effects as previously known dominant-interfering mutations were found. T cells from these patients that had been activated in vitro and cultured in IL-2 did not harbor the mutations, nor did the patients' buccal mucosal cells, indicating that the FAS defects had arisen by somatic mutation and were subject to positive selective pressure because of their resistance to physiological apoptosis signals in vivo. ALPS due to FAS defects arising by somatic mutation in T-cell precursors, common lymphoid progenitors, or hematopoietic progenitors is designated ALPS-sFAS (somatic mutant) (Table 30.2). A recent evaluation of 32 patients from the NIH cohort without germline mutations in *FAS* identified 50 percent of these individuals with a somatic mutation in *FAS* (Dowdell et al., 2010), and these findings establish somatic mutations in *FAS* as the currently second most common genetic basis for ALPS.

ALPS-LIKE ILLNESSES WITHOUT FAS DEFECTS

Both children and adults have been observed who have autoimmune lymphoproliferation but no mutations in *FAS*. Some of these individuals have modestly expanded numbers of DNT cells. Among this group are patients with impaired in vitro apoptosis via the FAS pathway; normal apoptosis after anti-FAS antibody exposure but impairment of apoptosis by TCR restimulation; impairment of apoptosis associated with cytokine (IL-2) withdrawal (with normal FAS-mediated cell death); and also patients with no demonstrable apoptosis defects (Dianzani et al., 1997; Oliveira et al., 2007; Sneller et al., 1997; S. E. Straus and J. M. Puck, unpublished observations; J. P. Villartay, unpublished observations). Additional patients with clinical findings similar to those of ALPS patients have failed to show elevations of DNT cells, although not all have been studied over long time periods with consistent staining protocols. A variety of patients with autoimmune blood cytopenias have splenomegaly with or without adenopathy; they are not considered to have ALPS unless the required features in Table 30.1 are fully met.

Of probands (the first subject enrolled in each kindred) in the NIH cohort who met the criteria for ALPS in Table 30.1, ~70 percent have either germline or somatic FAS defects (ALPS-FAS and ALPS-sFAS), as shown in Table 30.2. The remaining patients, however, had normal *FAS* gene sequences and were thus investigated for mutations in other components of lymphocyte apoptosis pathways, many of which are depicted in Figure 30.1. A single patient had a mutation in FASL, similar to the patient reported by Wu et al. (1996), and is now designated as having ALPS-FASL (Bi et al., 2001; Oliveira et al., 2010). No human cases to date have been published with absence of FADD, consistent with the finding that mice with targeted disruptions of this gene are nonviable.

Two heterozygous, dominant-interfering caspase 10 mutations have been found to cause ALPS-CASP10 in three families (Table 30.2; Wang et al., 1999; Zhu et al., 2006). The missense mutations are L285F, located in the proximal portion of the p17 protease domain, and I406L, very near the active site made up of conserved amino acids QACQG at

399–403. An additional mutation, V410I, was initially noted in homozygosity in a subject with recurrent fevers, noninfectious lymphocytic meningitis, optic neuritis, and no FAS defect (Wang et al., 1999). However, the patient's disease may have been in part caused by a subsequently identified TNF receptor-associated periodic fever mutation (see Chapter 33). Furthermore, the V410I variant of caspase 10 is now known to be a polymorphism in healthy populations, with an allele frequency of 6.8 percent in Danish, 3.4 percent in U.S. Caucasian, and 0.5 percent in African American unrelated individuals (Gronbaek et al., 2000; Zhu et al., 2006). Zhu et al. also confirmed that individuals with caspase 10 V410I, including homozygotes, are healthy and even found statistical evidence that caspase 10 V410I may have a protective effect against severe disease in subjects with *FAS* mutations that can cause ALPS-FAS. Zhu et al. further reported another caspase 10 population variant, Y446C, present in 1.6 percent of Caucasian alleles, for which no functional role has been demonstrated.

A single family has been found in which a son and daughter of a consanguineous marriage were homozygous for the caspase 8 mutation R248W, a nonconserved amino acid substitution in the proximal p18 protease subunit. Both children had features of ALPS, including adenopathy, mildly or intermittently increased DNTs, autoantibodies, and impaired lymphocyte apoptosis, while heterozygous relatives were unaffected. Moreover, unlike other ALPS cases, these children also manifested T- and B-cell immunodeficiency with growth retardation, recurrent sinopulmonary and herpes simplex virus infections, eczema, and poor responses to immunizations. The *CASP8* mutation they carried in homozygosity not only abrogated the cleavage activity of experimental protease substrates and failed to mediate apoptosis when transfected into epithelial cell lines; it also rendered the patients' lymphocytes refractory to activation in vitro (Chun et al., 2002). Further studies confirmed a requirement for full-length caspase 8 in the NF-κB activation phase of normal T-, B-, and NK-cell responses (Su et al., 2005). Thus caspase 8 has dual functions, in both apoptotic and cell activation pathways, and this is categorized as an ALPS-related disorder with specific designation of caspase 8 deficiency state (CEDS) (Oliveira et al., 2010).

ALPS-U patients are those who meet the two major criteria for ALPS as well as have one of the minor secondary criteria but do not have a defined genetic defect or a demonstrable in vitro FAS-mediated defect in cell death. It is possible that the majority of the patients described by Dianzani et al. (1997), who had ALPS with FAS pathway apoptosis defects but normal *FAS* gene sequences, also carry as-yet-unidentified mutations in other apoptosis pathway genes.

One patient within the NIH cohort who presented with findings suggestive of ALPS, but in whom the in vitro FAS-mediated cytotoxicity assay proved to be normal, was recently found to have a defect in cytokine (IL-2) withdrawal-mediated T-cell apoptosis. This finding was associated with decreased BCL-2 interacting mediator of apoptosis (BIM) levels and was ultimately identified as resulting from a somatic gain of function mutation in *NRAS* (Oliverira et al., 2007). Since this original description additional patients have been identified with similar findings including patients who had somatic gain of function mutations in *KRAS*, rather than *NRAS*, collectively these patients are now categorized as having RAS-associated autoimmune leukoproliferative disorder (RALD) (Oliveira et al., 2010, Niemela et al, 2011).

STRATEGIES FOR DIAGNOSIS

Unexplained splenomegaly and lymphadenopathy of early onset, particularly when accompanied by overt autoimmune disease and/or a positive family history, are the clinical features that lead to a suspicion of ALPS. More than 1.5 percent of α/β TCR $CD4^-$ $CD8^-$ T cells (among all lymphocytes, or 2.5 percent of T cells) are a defining characteristic of ALPS, but may not be counted correctly if only CD4, CD8, and CD3 are quantified, because several more common conditions are associated with increased γ/δ T cells lacking CD4 and CD8. Hypergammaglobulinemia, elevated numbers of B cells, and the presence of autoantibodies are further important clues and are more widely assayed. Since FAS membrane expression is absent only in rare patients with null mutations in both alleles, its assessment is of little help in diagnosis. Histological examination of enlarged lymph node biopsies yields a fairly characteristic appearance in ALPS, especially in cases associated with *FAS* mutations, and special stains revealing DNT cells can be diagnostic even in subjects who are not available to provide fresh blood samples (Lim et al., 1998). In vitro evaluation of lymphocyte apoptosis as triggered by agonistic FAS-specific antibodies can help establish the diagnosis.

Abnormal FAS-mediated apoptosis should lead first to investigation for causative mutations in the *FAS* gene transcript and sequence. Exon 9, the most frequently mutated portion of the gene, can be sequenced first, with subsequent attention to the remaining eight exons if no deleterious mutation is found. Special attention should be given to establishing the significance of missense mutations. SNPs in *FAS* have been delineated that are not associated with ALPS (Niemela et al., 2006). For previously unreported changes a panel of 100 chromosomes from 50 unrelated, healthy individuals helps to suggest that a missense mutation is not merely a polymorphism without functional consequences. However, functional assays of apoptosis should be combined with sequence abnormalities to prove the deleterious nature of any *FAS* mutation.

In a patient with clinical and immunological features of ALPS, a constitutional *FAS* mutation associated with defective FAS and impaired lymphocyte apoptosis would confirm the diagnosis of ALPS-FAS. Patients with FAS defects only in a sublineage of their hematopoietic cells, designated ALPS-sFAS, can be diagnosed on the basis of deleterious *FAS* mutations in isolated DNT cells (Dowdell et al., 2010; Holtzelova et al., 2004). If the sequence of both *FAS* alleles is normal, deleterious mutations in the genes encoding caspase 10 (ALPS-CASP10) can be sought, as well as FASL (ALPS-FASL) if apoptosis in response to anti-FAS stimulation is intact. Remaining patients without defined defects receive a diagnosis of ALPS-U.

It is not yet clear to what extent defects in FAS or other lymphocyte apoptosis mediators, particularly caspase 8, contribute to common variable immunodeficiency (CVID) (see Chapter 24) or autosomal hyper-IgM syndrome (see Chapter 21), both syndromes representing collections of defects with diverse genetic etiologies. Up to one third of CVID and autosomal hyper-IgM patients may experience chronic lymphadenopathy and/or splenomegaly, and autoimmune features are commonly present in both conditions. However, it is not common for CVID to be diagnosed as early in life as ALPS, and CVID frequently becomes more severe in the teen years to adulthood, when the findings of ALPS, if anything, tend to regress. Nonetheless, potential overlap between several features of ALPS and other primary immunodeficiency syndromes is an area for further investigation.

The differential diagnosis of ALPS versus malignancy and the apparent increased risk of lymphomas in ALPS remain major concerns for patients presenting with enlarged lymphoid organs or those who exhibit significant new lymph node enlargement. Biopsy of consistently large or rapidly growing nodes should reveal the characteristic histological features of ALPS (Plate 30.II), while in lymphoma a homogenous monoclonal cell population disrupts the normal architecture. Study of clonality of immunoglobulin or TCR gene rearrangements and cytogenetic investigation for chromosomal translocations must be done to identify true lymphomas in patients with a history of prolonged adenopathy.

FINDINGS IN RELATIVES OF ALPS PROBANDS

Studies of family members of ALPS-FAS (previously referred to as ALPS Ia) probands have been undertaken to find additional individuals who might carry the same mutations as ALPS probands (Bettinardi et al., 1997; Drappa et al., 1996; Fisher et al., 1995; Infante et al., 1998; Jackson et al., 1999; Rieux-Laucat et al., 1995, 1999; Sneller et al., 1997; Vaishnaw et al., 1999b). In some instances newly occurring *FAS* germline mutations have been found in probands whose healthy parents had normal *FAS* gene sequences (Sneller et al., 1997). In the rest of the families, whether the mode of inheritance was recessive or dominant, relatives with a mutated *FAS* allele have been identified. Some of these relatives proved completely free of symptoms and signs of ALPS, while others met the diagnostic criteria for ALPS (Table 30.1). Still other mutation-bearing relatives had some but not all of the features of ALPS, such as episodes of significant adenopathy or an enlarged spleen without autoimmune disease. For example, in the family of one ALPS patient followed by the NIH group, four relatives in addition to the patient were found to share the same mutation on one *FAS* allele. All had defective in vitro lymphocyte apoptosis. However, none of the relatives experienced any clinical manifestations of ALPS and none had autoantibodies or any other laboratory abnormalities except for one individual with minimally increased (1.7 percent) DNT cells.

In contrast, a large kindred was studied in which 12 individuals from four generations were followed for over 30 years with a variety of findings, including splenomegaly, adenopathy, and Coombs-positive anemia; affected subjects were recently proven to have a heterozygous mutation changing an aspartic acid to valine at position 244 (D244V) in the death domain of FAS (Fig. 30.4) (Infante et al., 1998). The diagnosis of ALPS was first confirmed in individual IV-5, who at age 2 was in general good health but had adenopathy and splenomegaly, Coombs-positive anemia, 2.1 percent DNT cells, and a plasma IgG of 2,355 mg/dL. All of the surviving mutation-bearing members of this kindred (black upper right quadrants of pedigree symbols) had impaired lymphocyte apoptosis. Furthermore, as shown, each of these individuals had features of ALPS in at least one category—lymphoproliferation, evidenced by splenomegaly and or adenopathy (lower right quadrant); 1.5 percent or more DNT cells (lower left quadrant); and autoimmunity, with either overt manifestations such as hemolytic anemia or ITP, or significant titers of autoantibodies (upper left quadrant). By late adolescence or

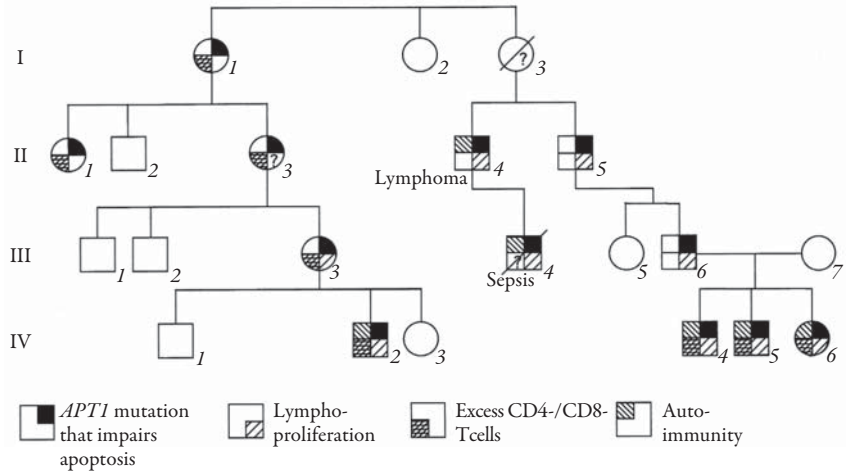

Figure 30.4 Dominant inheritance of *FAS* mutation and features of ALPS in a large kindred (adapted from Infante et al., 1998). Squares, males; circles, females; slash, deceased; ?, could not be ascertained.

adulthood, lymphoproliferation and autoimmune manifestations tended to abate, and the only premature death in the family was due to postsplenectomy sepsis at age 9 in individual III-4. However, the occurrence of NHL in individual II-4 at age 50, his development of ITP at age 23, and hemolytic anemia at age 54 demonstrate the lifelong risk of malignancy and the unpredictable course in this and many other families with FAS defects. Since this kindred was reported, Hodgkin's lymphoma was diagnosed in individual IV-4 at age 7 years.

Only limited family studies have been conducted in ALPS-CASP10, which, however, seems to have variable penetrance and severity similar to ALPS-FAS (Zhu et al., 2006). Prenatal diagnosis has not been published either by mutational analysis of *FAS* or by assessment of lymphocyte surface markers in fetal blood. The prenatal hydramnios of the severely affected recessive case reported by Le Deist et al. (1996) indicates that, at least in this patient, lymphoproliferation was ongoing before birth.

PROGNOSIS AND TREATMENT

Growing experience with ALPS indicates that patients can achieve a normal lifespan. Patient histories suggest that adenopathy may diminish with age; but while the most severe autoimmune disease appears to occur in early childhood, as indicated above, episodes can recur or in fact arise unpredictably at any age. In older individuals now recognized to have a longstanding history of ALPS, splenectomy was common, but recognition of the risks of infection in asplenic subjects has made this a treatment of last resort in current practice. Nonetheless, splenectomy has been performed in many of our patients with ALPS in order to manage their severe hypersplenism, severe hemolysis, or refractory thrombocytopenia. Splenectomy has also been performed following splenic rupture and in some cases when lymphoma was suspected. Fatal postsplenectomy sepsis has been documented in multiple patients despite prior administration of polysaccharide vaccines and prescription of prophylactic antibiotics. The current recommendation is to avoid splenectomy in ALPS patients if at all possible based on the observation that of 87 splenectomized patients followed at NIH, 5 have died. Two children had fatal sepsis due to *Streptococcus pneumoniae*, while three other patients developed opportunistic infections associated with multidrug immunosuppressive therapy for aggressive, infiltrative polyclonal lymphoproliferation (Rao et al., 2005).

Based on studies suggesting efficacy in *lpr* mice with an ALPS-like phenotype (Mountz et al., 1987), one patient received sequential therapeutic trials of prednisone, interferon-α, IL-2, and cyclosporine-A for treatment of her disease (Sneller et al., 1997). Therapy with high-dose prednisone resulted in a transient decrease of lymphadenopathy, but the lymph nodes returned to their original size following reduction of the dose. None of the other treatments resulted in a change in the degree of lymphadenopathy or clinical status. Many patients with ALPS suffer severe episodes of autoimmune hemolytic anemia, thrombocytopenia, and neutropenia requiring immunosuppressive therapy even after splenectomy. Most episodes of ITP and some hemolytic crises respond to short courses of high-dose steroids, such as 1 mg/kg of prednisone daily. In these patients lymph node size transiently decreases to some degree during treatment but increases in size after therapy. In several patients, multiple courses of high-dose steroid pulses, or prolonged daily steroid plus intravenous immunoglobulin therapy, have been used in an attempt to control the autoimmune disease. Attempts to treat patients with refractory autoimmunity using vincristine, azathioprine, methotrexate, and cyclophosphamide have not resulted in any clear indication that these agents are effective. Additional efforts to avoid the complications of chronic steroids and immunosuppressive regimens in children with ALPS have led to investigation of alternative agents.

The antimalarial drug combination of pyrimethamine and sulfadoxine (Fansidar) was reported to be effective in a preliminary study of seven ALPS patients, two with ALPS-FAS and five with ALPS-U (van der Werff Ten Bosch et al., 2002). However, experience with pyrimethamine (a component of Fansidar) in a study involving NIH patients did not substantiate the effectiveness of this agent in managing ALPS-related autoimmunity (Rao et al., 2007).

Mycophenolate mofetil (MMF) was evaluated in a group of NIH patients as a potential steroid-sparing immunosuppressive agent. MMF is a prodrug of mycophenolic acid, which inhibits a key enzyme in the purine synthetic pathway, an especially active pathway in T and B lymphocytes. MMF, previously shown to be effective in immune-mediated cytopenias (Hou et al., 2003; Howard et al., 2002), was used to treat autoimmune cytopenias in 13 ALPS patients who were steroid-dependent (Rao et al., 2005). Twelve of 13 responded, making possible reduction or termination of steroid treatment and in some cases avoidance of splenectomy, and a second report of successful MMF therapy confirmed its utility (Kossiva et al., 2006).

More recently rituximab was used in 12 ALPS patients with autoantibodies to red cells and/or platelets associated with refractory cytopenias. Seven of nine adult patients responded, but none of the three children who were treated showed improvement with this regimen (Rao et al., 2009). Finally, based on the effectiveness of rapamycin therapy in the murine model of ALPS (Teachey et al., 2006), four ALPS patients were treated with rapamycin, and in three of four patients there was resolution of the autoimmune cytopenias as well as decreased lymphadenopathy and DNT cells (Teachy at al., 2009). These encouraging results await additional experience to confirm the utility of rapamycin therapy in ALPS.

Most ALPS patients can be weaned from treatment after an acute episode of an autoimmune cytopenia, and the rate of recurrence and severity of autoimmune attacks may diminish as patients enter adolescence. Adults with ALPS may cease to experience autoimmune complications, but conversely, new onset of ITP has occurred in adulthood (Drappa et al., 1996; Infante et al., 1998).

In a patient with complete FAS deficiency, ALPS manifestations were so severe that several courses of antithymocyte globulin and then chemotherapy were given to reduce the lymphocyte burden (Le Deist et al., 1996). This treatment

proved to be only transiently beneficial, so ultimately a T-cell–depleted, parental haploidentical bone marrow transplantation (BMT) was undertaken. A first attempt of BMT led to early rejection. However, a second transplant using T-cell–depleted marrow from the second HLA haploidentical parent was successful. There was no recurrence of lymphoproliferation or of autoimmunity, although $CD4^-$ $CD8^-$ T cells were detected in the child's blood for over 2 years. This child was well 3 years after the second BMT attempt (Benkerrou et al., 1997). Clearly, the role of BMT is not well defined in ALPS, and this treatment should be considered only for the rare patient in whom life-threatening complications prove refractory to more conservative measures.

ANIMAL MODELS

The MRL *lpr* mouse pointed the way to the discovery of FAS defects in humans. This spontaneous mouse mutant strain has increased autoimmunity compared to the MRL parent strain; it was discovered to have high numbers of $CD4^-$ $CD8^-$ lymphocytes and defective apoptosis and was subsequently demonstrated to be Fas-deficient (Nagata and Goldstein, 1995; Watanabe-Fukunaga et al., 1992). Originally described as having a lupus phenotype, the MRL *lpr* mouse actually lacks some of the more typical features of human lupus such as anti-DNA antibodies and frequent arthritis. However, these mice regularly develop antinuclear antibodies, hypergammaglobulinemia, adenopathy, and nephritis. Additional spontaneous mutant mouse strains with similar phenotype are *gld*, found to have recessive defect of Fas ligand, and lpr^{cg}, a strain with a death domain point mutation causing a change in a conserved amino acid of Fas. Transgenic mice expressing dominant negative mutations in Fas corresponding to the mutations found in humans with ALPS had apoptosis defects and developed hepatosplenomegaly and lymphocytic liver infiltration (Choi et al., 1999).

Like the human, the phenotype in mice with *lpr* mutation is highly strain-dependent. For example, C57BL6 mice with the *lpr* mutation have much less severe disease than MRL*lpr* mice. Furthermore, the MRL strain is autoimmune-prone even without defective apoptosis conferred by the *lpr* Fas defect, in part due to a hypoactive variant of IL-2 (Choi et al., 2002). Both class I and class II major histocompatibility complex (MHC) determinants are important in development of autoimmunity in Fas-deficient mice (Christianson et al., 1996; Creech et al., 1996).

The range of autoimmune findings in *lpr*, *gld*, and lpr^{cg} mice is not identical to that seen in humans with ALPS; for example, nephritis is uncommon in patients, although isolated cases have been seen. While FASL defects are not a frequent cause of human ALPS, an adult male with atypical lupus and adenopathy was reported to have a dominant heterozygous *FASL* mutation (Wu et al.; 1996), an ALPS patient in the NIH series had a FASL defect (Bi et al., 2001) and recently an additional ALPS patient was described with a homozygous null FASL mutation (Magerus-Chatinet et al., 2013).

Although the mouse is an excellent model system in which to evaluate relationships between Fas apoptosis and other modulators of immune responses, differences between species need to be recognized. For example, the mouse lacks caspase 10, which in the human genome is located adjacent to caspase 8 and probably arose by gene duplication. Thus the relative roles of these caspases in the Fas apoptosis pathway cannot readily be dissected in mice. Mice with targeted disruptions of caspase 8 have cardiac and hematopoietic defects and do not survive (Varfolomeev et al., 1998), while humans lacking caspase 8 activity have features of both ALPS and combined immunodeficiency.

FUTURE DIRECTIONS AND CHALLENGES

MOLECULAR EVENTS CONTROLLING APOPTOSIS

The importance of programmed cell death in diverse physiological processes involving dividing cells is widely recognized. We now understand that apoptosis is critical for maintenance of immune-cell homeostasis, both by terminating an appropriate immune response and by preventing runaway responses by mature lymphocytes with cross-reactivity to self-antigens. However, all of the molecular events that deliver an apoptotic signal, the process of cell death itself, and the regulation of every step of the process are not yet fully understood (Chinnaiyan and Dixit, 1997; Nagata, 1997). Added insights into these processes have been garnered with extensive studies driven by the availability of cells with FAS defects and of increasingly sophisticated in vitro technologies. For example, the redistribution of FAS into lipid rafts at the cell surface has been highlighted as an important mechanism for making lymphocytes more sensitive to apoptosis (Muppidi and Siegel, 2004). The transmembrane molecules TNFR-1 and FAS interact with their ligands as trimers and share intracellular death domain regions. Both the TNFR-1 pathway and the FAS pathway are expressed in mature lymphocytes (Zheng et al., 1995). The TNFR-1 pathway involves some genes with restricted specificity, such as TNFR-1 itself, TRADD, and TRAF-2, but some of the other death domain interacting genes and perhaps certain caspases and final nuclear effectors of apoptosis may be shared with the FAS pathway. An additional apoptosis pathway through a distinct receptor DR3 (death receptor 3) has been identified in lymphocytes, and additional receptors DR4 and DR5 have been isolated (Chinnaiyan et al., 1996). Other intracellular signal transducers are also involved. The pivotal molecule NF-κB can be activated and appears to have an antiapoptotic effect by upregulating cellular survival factors, but its relationship with caspase 8 in cell activation is also essential. It is not entirely clear how mitochondrial apoptosis pathways interact with pathways of apoptosis involving TNFR family members, and defects in these pathways underlie some cases of ALPS-U as well as other clinical syndromes involving inflammation, lymphoproliferation, and immune dysregulation.

MODIFIERS OF THE ALPS PHENOTYPE

Even among ALPS patients with a well-defined FAS defect, the clinical phenotype can be highly variable, as appreciated early in the course of work in the field, suggesting that additional genetic and/or environmental factors must be required to interact with FAS defects to produce overt lymphoproliferation and autoimmunity. These putative ALPS-modifying factors might include other members of the FAS apoptosis pathway or related pathways, many of which are continuing to be identified. An existing model for severe disease produced by complementation between two apoptosis pathway mutations is the mouse doubly heterozygous for *gld* and *lpr*^{cg} defects of FasL and Fas, respectively. Identification of critical intracellular signaling mediators in the caspase protease cascade provides additional candidate genes for modifiers of the ALPS phenotype. Moreover, a large number of gene products regulating lymphocyte signaling networks have been implicated in lymphoproliferation and autoimmunity in mice; many of these could be exacerbated by further impairment of apoptosis and therefore may also be candidates for second mutations in ALPS.

The MHC locus is a major modifier of risk for many autoimmune conditions, and preliminary studies indicate that HLA B44 may be protective against severe disease in individuals with ALPS-FAS (Vacek et al., 2006). The caspase 10 variant V410I appears to be protective (Zhu et al., 2006). In contrast to many autoimmune diseases, ALPS appears to affect males equally or even to a greater degree than females. Identifying and understanding the mechanisms whereby these and other factors modify risk promises to clarify the genetic etiologies of further autoimmune syndromes, and perhaps to provide new therapeutic approaches for autoimmune disorders.

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31.

AUTOIMMUNE POLYGLANDULAR SYNDROME TYPE 1

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Autoimmune diseases arise from a breakdown in immune tolerance to self, and there are numerous genes and pathways that play a role in autoimmune susceptibility (Rioux and Abbas, 2005). Most autoimmune diseases arise from a complex interplay between environmental and genetic factors, but recent work on monogenic forms of autoimmunity has been particularly enlightening in expanding our understanding of how these diseases arise (Sakaguchi et al., 2008; Su and Anderson, 2009). Among these monogenic disorders is autoimmune polyglandular syndrome type 1 (APS1) or autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), which is manifested by a pattern of autoimmunity that usually involves multiple organs. In this review, we will highlight key aspects of the clinical features and update the latest in our understanding of the underlying pathogenesis of this syndrome.

CLINICAL MANIFESTATIONS

APS1 is a rare autosomal recessive disorder that was originally described in a case report by Thorpe and Handley in 1929 (Thorpe and Handley, 1929); later on, the strong familial inheritance of the syndrome was recognized (Whitaker et al., 1956). The disorder has been found in many different populations and genetic backgrounds, but there are isolated areas/populations where the disease is more frequently observed, particularly in Finland, Sardinia, and Iranian Jews, with prevalence estimates of 1/25,000, 1/9,000, and 1/14,000 respectively (Ahonen et al., 1990, Rosatelli et al., 1998, Zlotogora and Shapiro, 1992). The clinical manifestations of APS1 are somewhat variable even within families, but prominent features include the triad of hypoparathyroidism, Addison's disease, and susceptibility to mucocutaneous candidiasis (Plate 31.I, 31.II). The presence of two of these three features, in fact, is considered by many to be diagnostic of the disorder (Perheentupa, 2006). However, there are also affected patients who do not manifest two of these features (Buzi et al., 2003). In addition to these major features, a wide number of autoimmune diseases have been described in APS1 patients, including autoimmune hepatitis, vitiligo Plate 31.III, alopecia Plate 31.III, premature gonadal failure, hypothyroidism, type 1 diabetes, autoimmune gastritis, uveitis Plate 31.IV, Sjögren syndrome, and autoimmune hepatitis (Betterle et al., 1998, Perheentupa, 2002, Ahonen, 1985). In addition to these autoimmune features, patients also develop other clinical findings that may or may not be autoimmune sequelae, including dental enamel hypoplasia Plate 31.IV, tympanic membrane calcification, pitted nail dystrophy (Plate 31.I Plate 31.IV) and intestinal malabsorption. We have summarized many of the clinical disorders described in these patients in Table 31.1 and organized them by their overall penetrance in relation to descriptions in the literature. Generally, it appears that many of the manifestations of the syndrome involve an organ-specific autoimmune attack rather than systemic autoimmunity, as descriptions of systemic lupus erythematosus and rheumatoid arthritis in these patients are exceedingly rare. There is also a relative pattern to the timing of when the disease features arise in these patients, with onset of one of the defining features often in early childhood; however, some patients do not manifest until early adulthood (Betterle et al., 1998; Perheentupa, 2006). Typically, candidal infection is the first manifestation, followed by hypoparathyroidism, and then Addison's disease, but again there can be wide variation in the timing and order. Given the wide variety of potential features associated with this syndrome, the clinical care and workup of these patients is complex and laborious. Perheentupa et al. have some of the most extensive experience in the expert care of these patients and have written a number of excellent reviews with extensive detail on the phenotypes, markers, and clinical care issues

Table 31.1 CLINICAL MANIFESTATIONS OF APS1

PENETRANCE		
HIGH	INTERMEDIATE	RARE BUT DESCRIBED
Candidiasis	Uveitis	Asplenia
Hypoparathyroidism	Ovarian failure	Pituitary insufficiency
Addison's disease	Type 1 diabetes	Hyperthyroidism
	Hypothyroidism	Autoimmune hemolytic anemia
	Alopecia	Sjögren syndrome
	Vitiligo	Nephritis
	Pernicious anemia/gastritis	Retinal degeneration
	Autoimmune hepatitis	Lymphocytic myocarditis
	Diarrhea/malabsorption	Interstitial lung disease
	Enamel hypoplasia	Autoimmune peripheral neuropathy
	Tympanic membrane calcification	

Although a number of extensive features are listed, it is by no means a complete listing for all APS1 features.

REFERENCES: Alimohammadi et al., 2009; Betterle et al., 1998; Perheentupa, 2002, 2006; Valenzise et al., 2009.

(Perheentupa, 2002, 2006). Here we highlight a few details of the major features of the disorder.

Susceptibility to candidiasis is one of the most prominent features of the disease, and it most often presents with oral/pharyngeal infection in these subjects (Perheentupa, 2002). Candidal infection involving the gastrointestinal tract, including perianal involvement, has been described. Postpubertal candidal vulvovaginitis is also quite common. Some subjects can also demonstrate infection of the digits of both the hands and feet. Despite this remarkable susceptibility pattern to *Candida*, it is important to note that these patients do not generally demonstrate susceptibility to systemic candidal infections. Treatment of candidal involvement typically involves courses of oral or topical antifungal agents, and patients usually show a response to this approach. Occasionally patients may require systemic antifungal treatment with persistent infections. No clear diagnostic testing is currently available to determine the cause of this susceptibility or to identify subjects with increased susceptibility, but there are descriptions of anergic skin testing to candidal antigens in APS1 subjects (Chilgren et al., 1967; Tomar et al., 1979). Finally, it is important to note that it is likely that the chronic oral involvement by *Candida* is likely carcinogenic, as an association with epithelial carcinoma of the oral mucosa has been noted (Perheentupa, 2006).

Hypoparathyroidism is also a common feature observed in APS1. Typically patients present with a vague clinical course that may include episodes of tetany or even grand mal seizures. Serum testing usually reveals a low serum calcium level and hyperphosphatemia in the setting of a low or inappropriately normal parathyroid hormone level. Treatment is calcium replacement along with vitamin D supplements. Care must be taken not to allow the serum calcium level to become too high, as this can often precipitate nephrolithiasis due to poor calcium reabsorption from the lack of parathyroid hormone. Although there have been reports of autoantibodies to the calcium-sensing receptor as a marker of this disease phenotype, data have been conflicting (Alimohammadi et al., 2008; Gavalas et al., 2007; Soderbergh et al., 2004). There has been recent identification of a novel autoantigen, NALP5, that may be a marker of this disorder (see discussion below) (Alimohammadi et al., 2008).

Addison's disease or autoimmune adrenal failure is a common feature in these subjects that also has an occult clinical course. Patients can present with malaise, fatigue, salt wasting, and hypotension as the disease progresses. Diagnosis is usually made by clinical suspicion and failure to secrete increased cortisol after stimulation with ACTH (Cortrosyn). In addition, autoantibodies to 21-hydroxylase (CYP450c21), an established marker of autoimmune adrenal failure, are usually present in these patients. This marker also appears before patients progress to adrenal insufficiency and is likely to be predictive for progression to adrenal failure within months to several years (Perheentupa, 2002). Affected patients are usually treated with cortisol and mineralocorticoid replacement, with instructions for stress dosing of cortisol during times of infection and additional stress.

Although less prevalent, APS1 patients develop a number of other endocrine deficiencies, particularly type 1 diabetes, hypothyroidism, and premature ovarian failure. The incidence of type 1 diabetes in APS1 cohorts is variable but reported to be as high as 18 percent, supporting that these patients have an increased susceptibility (Perheentupa, 2002). Interestingly, it appears that a much larger number of APS1 patients harbor autoantibodies to the type 1 diabetes autoantigen glutamic acid decarboxylase (GAD) than in isolated type 1 diabetes. Hypothyroidism is moderately apparent in these patients and is also associated with the typical marker autoantibodies to thyroid peroxidase and thyroglobulin. Finally, it is worth noting that many female patients develop premature ovarian failure, which is usually associated with low estrogen levels in the setting of high gonadotropin levels.

One potential manifestation of the disorder that requires particular attention is autoimmune hepatitis. This is because it is one of the few features of the disease that is treated with systemic immunosuppression with good clinical efficacy, and if

left untreated subjects can progress to fulminant liver failure. Abnormal liver function test results should raise suspicion, and the diagnosis is usually confirmed by needle biopsy of the liver. Patients do manifest autoantibodies seen in a subset of autoimmune hepatitis patients, particularly antibodies to liver microsomal antigen (Obermayer-Straub et al., 2001).

Overall, as mentioned above, the care of these patients is complex and involves rigorous surveillance for many of the potential features of the disease. Typically, treatment involves hormone replacement for endocrine deficiencies and immunosuppression if autoimmune hepatitis develops. Long-term data on these patients suggest that they do have increased mortality even under expert care, and there is likely a significant risk for depression and suicide beyond the autoimmune manifestations of the disorder.

GENETICS OF APS1 AND THE AIRE PROTEIN

APS1 is rare among autoimmune diseases in that it appeared to be inherited in a monogenic, autosomal recessive fashion. In 1997, using large family collections, two groups independently identified the *A*uto*i*mmune *Re*gulator (Aire) as the defective gene responsible for APS1 (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997). This gene encodes a 545 amino acid, 58 kDa protein and is located on chromosome 21q22.3. Aire was an intriguing candidate in that its expression pattern predominantly in the thymus (and to a lesser extent in the lymph node and spleen) was highly suggestive of a role in immune function.

Additionally, a number of features suggest a role for Aire as a transcription factor. First, endogenous Aire protein is located in the nucleus in mouse thymi (Liston et al., 2004). The Aire protein contains a nuclear localization signal (NLS) that is sufficient to target the Aire protein to the nucleus (Pitkanen et al., 2001), and the recently described caspase-recruitment domain (CARD) also appears to have a role in a nuclear localization (Ferguson et al., 2008; Fig. 31.1).

Second, Aire contains a number of domains that suggest a role in transcriptional regulation (Fig. 31.1), including two plant homeodomains (PHDs) domains and a SAND (Sp100, Aire-1, Nucp41/75, and DEAF-1) domain. The PHDs have been described in a large number of transcription factors and have recently been implicated in the binding of nucleosomal histones. For example, the PHD in the bromodomain PHD finger transcription factor (BPTF) and inhibitor of growth family, member 2 (ING2) proteins has been shown to bind to histone H3 trimethylated at lysine 4 (H3K4me3) (Li et al., 2006; Shi et al., 2006). As discussed later, the PHD domain of Aire itself binds to histone H3 unmethylated at lysine 4 (H3K4me0) (Koh et al., 2008, Org et al., 2008).

In the Sp100b and glucocorticoid modulatory element binding protein 1 (GMEB) proteins, the SAND domain binds directly to DNA via the KDWK motif (Bottomley et al., 2001; Surdo et al., 2003). The Aire SAND domain, however, does not contain this KDWK motif, suggesting that the SAND domain of Aire may have a different function. Although there have been a small number of reports of Aire's direct binding to DNA (Kumar et al., 2001; Purohit et al., 2005; Ruan et al., 2007), these findings have not been widely replicated. Thus, the role of the SAND domain of Aire remains to be further studied.

Intriguingly, a point mutation in the Aire SAND domain that changes glycine 228 (in humans) to a tryptophan has been shown in both humans and mice to result in an autosomal dominant form of autoimmunity (Cetani et al., 2001; Su, 2008). This autoimmunity is phenotypically distinct from that seen with homozygous null mutations in Aire, resulting in largely autoimmune thyroiditis in humans. This mutation appears to act in a dominant negative fashion, preventing transcriptional transactivation by wild-type Aire in in vitro assays. These findings raise the possibility that some autoimmune diseases that do not appear to be APS1 may result from Aire mutations.

PATHOPHYSIOLOGY

Much of the pathophysiology of APS1 has been worked out in mouse models of this disease. In recent years, several different lines of Aire knockout mice have been independently generated (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002). Like humans with homozygous

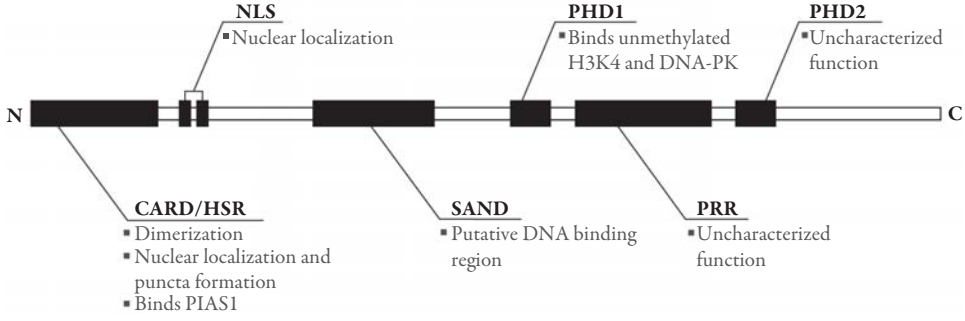

Figure 31.1 *Schematic of the Aire protein and its domains.* Black text indicates domain name, with gray text underneath highlighting known or potential function roles. NLS, nuclear localization signal; PRR, proline-rich region; HSR, homogeneous staining region; CARD, caspase-recruitment domain; SAND, Sp100, Aire-1, Nucp41/75, and DEAF-1; PHD, plant homeodomain.

mutations in Aire, these knockout mice develop spontaneous, organ-specific autoimmunity.

A clue into how Aire works came from its predominant expression pattern in the thymus. In particular, Aire appeared to be expressed in a subset of stromal cells in the thymus termed medullary thymic epithelial cells (mTECs) (Halonen et al., 2001; Heino et al., 1999; Zuklys et al., 2000). mTECs have been described to play a major role in the negative selection of self-reactive T cells in the thymus (reviewed in Kyewski and Klein, 2006). mTECs have the extraordinary property of expressing a number of tissue-specific antigens, including insulin and thyroglobulin (Smith et al., 1997). These antigens classically have been thought to be expressed only in an organ-specific manner (i.e., insulin in the pancreas and thyroglobulin in the thyroid). The "promiscuous" expression of these antigens in mTECs has been proposed to allow developing T cells in the thymus to sample self-antigens and allow self-reactive T cells to be eliminated.

Because Aire appeared to be a transcription factor, its expression pattern in mTECs led to the hypothesis that Aire was important in upregulating the expression of tissue-specific antigens in mTECs (Fig. 31.2A). Thus, loss of Aire would result in decreased expression of these antigens in mTECs and allow self-reactive T cells to escape negative selection in the thymus and potentially cause autoimmune disease (Fig. 31.2B). This hypothesis was validated in the Aire knockout mouse system, where isolated mTECs from these mice showed decreased expression of a number of tissue-specific antigens, including insulin (Anderson et al., 2002). Consistent with this hypothesis, loss of expression of a single eye-specific antigen in mTECs has been shown to result in autoimmune uveitis in mice (Devoss et al., 2006). Additionally, using transgenic mouse lines that allow the tracking of antigen-specific T cells, Aire-deficient mice appear to have a failure in negative selection of developing thymocytes that react against self-antigens (Anderson et al., 2005; Liston et al., 2003).

Interestingly, Aire is also expressed in secondary lymphoid organs, including bone marrow and spleen (Gardner et al., 2008). Like mTECS, these extrathymic Aire-expressing cells (eTACs) also express a number of tissue-specific antigens. The overlap of these antigens with those expressed by mTECs is surprisingly small, suggesting that these cells may play a nonredundant function in maintaining self-tolerance. For example, desmoglein, the antigen important in pemphigus foliaceus, is expressed in eTACS but not in mTECs. However, the physiological role of these cells in preventing autoimmunity remains to be tested.

How Aire is seemingly able to upregulate the expression of a large array of genes has been the subject of much interest. It has been proposed that Aire-regulated genes appear to be physically clustered on chromosomes (Derbinski et al., 2005; Johnnidis et al., 2005), which may perhaps facilitate their regulation as a group by Aire. These clusters of Aire-regulated genes, however, are often interspersed with Aire-independent genes, suggesting a more complex mechanism by which Aire is acting.

On a subcellular level, Aire is closely associated with nuclear speckles (Su et al., 2008). These nuclear speckles contain

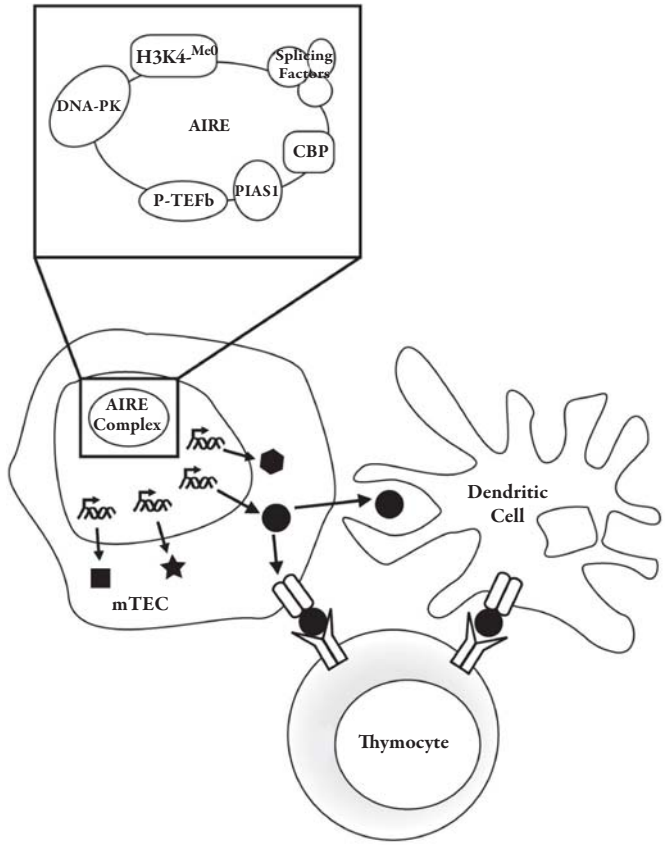

Figure 31.2 *Functional activity of Aire in thymic medullary epithelial cells (mTECs).* Aire likely works in a complex with an array of other nuclear proteins (including DNA-PK, P-TEFb, PIAS1, CBP, and other splicing factors) to drive the expression of a wide array of tissue-specific antigens (indicated by circle and star) within mTECs. These tissue-specific antigens can be processed and displayed in a MHC/self-antigen complex on the surface of mTECs or alternatively passed on to nearby dendritic cells for MHC/self-antigen complex formation. This MHC/self-antigen complex is then displayed to developing thymocytes to negatively select out those that are autoreactive. DNA-PK, DNA protein kinase; P-TEFb, positive transcription elongation factor b; PIAS1, protein inhibitor of activated STAT 1; CBP, CREB binding protein.

proteins important in transcription, including small nuclear ribonuclear proteins (snRPs), RNA polymerases, and splicing factors (Lamond and Spector, 2003). Additionally, Aire contains two PHD domains, which have been shown in other proteins to interact with chromatin (reviewed in Peterson et al., 2008). These zinc-finger–containing domains specifically seem to interact with histone H3, a core nucleosome protein that is subject to epigenetic modifications with methyl groups. Aire appears to bind to demethylated histone H3 (H3K4me0) via the PHD1 domain (Koh et al., 2008; Org et al., 2008).

In addition to demethylated histone H3, a number of other binding partners for Aire have also been described. These binding partners include positive transcription elongation factor b (pTEFb) (Oven et al., 2007), CREB binding protein (CBP) (Pitkanen et al., 2000), protein inhibitor of activated STAT 1 (PIAS1) (Ilmarinen et al., 2008), and DNA-dependent protein kinase (DNA-PK) (Liiv et al., 2008; Fig. 31.2). The mechanism by which Aire acts as a transcriptional regulator is the subject of much investigation at this point, and much

of how these binding partners may participate in Aire function remains to be determined. Nevertheless, all of these binding partners have potential roles in transcription (reviewed in Peterson et al., 2008). The pTEFb complex phosphorylates RNA polymerase II, allowing for the polymerase to convert to the elongating form and for transcription to proceed. CBP is a cofactor for a number of different transcription factors and is involved in transcription regulation. PIAS1 and DNA-PK bind to DNA sequences in the nuclear matrix, which may be an important site for chromatin assembly.

The tissues targeted in Aire-deficient mice appear to be dependent on mouse strain, pointing to a role for modifier genes in determining the disease phenotype (Jiang et al., 2005; Niki et al., 2006). Modifier genes also appear to play an important role in determining disease phenotype in humans, since the major histocompatibility complex (MHC) locus appears to have an effect on expression of disease (Gylling et al., 2000; Halonen et al., 2002). While some of the MHC associations overlap with those seen with the isolated autoimmune disease, others do not. For example, the risk alleles that confer susceptibility to type 1 diabetes do not appear to increase the likelihood of type 1 diabetes in APS1 patients.

In addition to upregulating transcription of a variety of tissue-specific antigens, Aire has also been proposed to have additional roles. These roles include regulating antigen presentation on the cell surface of mTECS (Anderson et al., 2005), regulating B-cell responses (Lindh et al., 2008), and in the differentiation of mTECs (Gillard et al., 2007). These roles for Aire remain to be further defined.

RECENT ADVANCES IN THE EVALUATION OF APS1

As discussed above, the classic triad of APS1 consists of hypoparathyroidism, adrenal insufficiency, and mucocutaneous candidiasis. Mucocutaneous candidiasis is a highly penetrant finding, with all adult patients in a cohort of 91 Finnish patients having manifested this disease (Perheentupa, 2006). Hypoparathyroidism and adrenal failure were also quite penetrant, with 88 percent and 84 percent of APS1 patients affected, respectively. In addition, a number of other endocrine and nonendocrine manifestations are present at variable frequency. It is important to note that in this cohort as well as others, there was great variability in the disease presentation of APS1 even among siblings.

In patients suspected of having APS1 by clinical findings, a number of laboratory evaluations can be useful adjuncts. First, DNA sequencing of the Aire locus can confirm the diagnosis. The most common mutations described are the R257X mutation and a 13 base pair deletion in exon 8. Importantly, however, approximately 60 mutations have been described throughout the Aire gene (Peterson et al., 2008). Thus, if the most common mutations are not found in a patient suspected of having APS1, sequencing of the entire gene may be necessary.

Second, autoantibody testing may be useful in helping to determine organs affected or at risk for being affected. Much effort has recently been focused on identifying the particular antigens important in autoimmune pathogenesis in APS1. Recently, autoantibodies against the NALP5 {NACHT [*NA*IP (neuronal apoptosis inhibitory protein), *C*IITA (MHC class II transcription activator), *H*ET-E (incompatibility locus protein from *Podospora anserina*), and *T*P1 (telomerase-associated protein)] leucine-rich-repeat and pyrin protein 5} antigen have been identified as a potential clinically useful marker for identifying APS1 patients with autoimmune hypoparathyroidism (Alimohammadi et al., 2008). NALP5 autoantibodies appear to be specific for APS1 patients with hypoparathyroidism, since none of the patients with isolated hypoparathyroidism appeared to have these autoantibodies. Interestingly, autoantibodies against calcium-sensing receptor were negative in all APS1 patients with hypoparathyroidism in this study.

Additional headway toward antigen identification has been made in Aire-deficient mice. As noted earlier, the expression of the IRBP (interphotoreceptor retinoid-binding protein) eye antigen in the thymus was shown to be sufficient in preventing eye-specific autoimmunity (Devoss et al., 2006). These mice develop autoantibodies against IRBP, suggesting that these autoantibodies may be a useful marker of uveitis in patients. These studies in APS1 patients with uveitis remain to be performed. Additional antigens identified in Aire-deficient mice include SVS2 (seminal vesicle secretory protein 2) in autoimmune prostatitis (Hou et al., 2009) and mucin in autoimmune gastritis (Gavanescu et al., 2007).

Third, antibodies directed against type 1 interferons have been shown to associate strongly with having mutations in Aire. In five separate studies, almost all patients with Aire mutations appear to have type 1 interferon autoantibodies (Meager et al., 2006; Meloni et al., 2008; Wolff et al., 2007; Zhang et al., 2007). Although the pathogenesis and consequences of these antibodies are not clear, testing for these antibodies may be a useful tool in diagnosing APS1. These antibodies may, for example, help to diagnose a suspected patient for whom one or both Aire mutations are not found. It must be noted, however, that these antibodies are not specific only to APS1, and patients with diseases like myasthenia gravis with thymoma may also have type 1 interferon autoantibodies (Meager et al., 2003).

TREATMENT FOR APS1

At this point in time, treatment for APS1 is largely supportive for the endocrine dysfunction seen in APS1. This includes hormone replacement for endocrinopathies, including adrenal failure, type 1 diabetes, hypothyroidism, and growth hormone deficiency; and vitamin D, calcium, and potentially magnesium supplementation for hypoparathyroidism. Dr. Perheentupa has noted in his extensive clinical experience in Finland that treatments for hypoparathyroidism, adrenal failure, and diabetes mellitus can interact with each other, complicating the management of APS1 patients (Perheentupa, 2006). Immunosuppression has been used to treat the nonendocrine manifestations of APS1, including autoimmune hepatitis.

Importantly, the mucocutaneous candidiasis seen in APS1 is a significant cause of morbidity and mortality and should

be treated aggressively. In a cohort of 91 Finnish patients, mucocutaneous candidiasis resulted in a number of complications, including esophageal strictures, candidal abscesses, and squamous cell carcinoma of the mouth and esophagus (Perheentupa, 2006).

In mouse models of disease, attempts have been made to try to alter the immune function in Aire-deficient animals as a way of curing the disease. In mice, both anti-CD4 antibody (Devoss et al., 2008) and anti-CD20 (Gavanescu et al., 2008) treatments have been used to prevent disease progression. As far as immunotherapy in patients, there are sparse data as to the efficacy of immunotherapeutic approaches toward the disease as a whole. In one case report, a 13-year-old patient with APS1 appears to have responded to an 8-month course of oral cyclosporine, with improvement in exocrine pancreatitis, keratoconjunctivitis, alopecia, and hepatitis (Ward et al., 1999). Without a systematic, controlled trial, however, it is difficult to assess if this treatment actually prevented or ameliorated disease. Importantly, side effects of the treatment included hypomagnesemia, hyperuricemia, and decreased glomerular filtration rate. Given the rarity of APS1, however, systematic trials of immunotherapies may be difficult to carry out.

AREAS FOR FUTURE STUDY

APS1 has classically been thought of as an autoimmune disorder. However, given the predisposition of patients to mucocutaneous candidiasis, APS1 can also be thought of as a state of immune deficiency. Thus, like many syndromes with immune manifestations (either autoimmunity or immune deficiency), APS1 should perhaps be thought of most accurately as a syndrome of immune *dysregulation*.

Much of the pathophysiology of APS1 has been recently unraveled. However, a number of areas remain open for further study: how the findings in mice translate to humans; the mechanism by which Aire is influencing the gene transcription of a large number of tissue-restricted antigens; and the pathophysiology underlying the immune deficiency and non-autoimmune manifestations are just some of the areas that will be unraveled in future studies.

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32.

IMMUNE DYSREGULATION, POLYENDOCRINOPATHY, ENTEROPATHY, AND X-LINKED INHERITANCE

Troy R. Torgerson, Eleonora Gambineri, and Hans D. Ochs

In 1982, Powell et al. reported on a large family with multiple affected males in a five-generation pedigree (Fig. 32.1). Affected males presented early in life with multiple endocrinopathies, severe chronic enteropathy, dermatitis, autoimmune hemolytic anemia, and antibody-induced neutropenia and thrombocytopenia. The syndrome was named IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked). Most of the affected boys died before the age of 3 years due to malabsorption, failure to thrive, infections, or other complications. Characteristic findings at autopsy of these and subsequently identified patients included lymphocytic infiltrates in the lungs and endocrine organs, particularly the pancreas and thyroid, and increased lymphoid elements in lymph nodes and spleen (Powell et al., 1982; Wildin et al., 2001). Symptomatic therapy with immunosuppressive drugs provided limited beneficial effects (Seidman et al., 1990).

Some 20 years before the initial description of this syndrome, a spontaneously occurring mutant mouse strain called *scurfy* was identified at the Oakridge National Laboratory (Russell et al., 1959). Affected mice have many phenotypic similarities to IPEX, including X-linked inheritance, dermatitis, and a wasting syndrome caused by enteropathy. Death occurs invariably during the first 3 to 4 weeks of life.

In humans, the gene responsible for IPEX was mapped to Xp11.23–Xq13.3 by linkage analysis (Bennett et al., 2000; Ferguson et al., 2000)., Shortly thereafter, the gene responsible for the scurfy syndrome in mice was identified by positional cloning as a transcription factor belonging to the forkhead/winged helix family and designated *Foxp3* (Brunkow et al., 2001). The human ortholog, *FOXP*3, was subsequently recognized as the causative gene for IPEX syndrome (Bennett et al., 2001b; Chatila et al., 2000; Wildin et al., 2001). Following this discovery, additional families with mutations in *FOXP*3 were identified and the full extent of the clinical phenotype of IPEX (OMIM304930) began to be clearer (An et al., 2011; Bae et al., 2011; Fuchizawa et al., 2007; Gambineri et al., 2003; Halabi-Tawil et al., 2009; Harbuz et al., 2010; Hashimura et al., 2009; Kobayashi et al., 2001; McGinness et al., 2006; McMurchy et al., 2010; Moes et al., 2010; Moraes-Vasconcelos et al., 2008; Nieves et al., 2004; Ochs et al., 2007; Ohshima et al., 2009; Otsubo et al., 2011; Owen et al., 2003; Torgerson et al., 2007; Wildin et al., 2002).

Finally, using the scurfy mouse model, three independent groups demonstrated that Foxp3 plays an essential role in generating $CD4^+CD25^+$ regulatory T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), providing convincing evidence that the lack of regulatory T cells is the direct cause of the early-onset autoimmunity observed in scurfy mice and patients with the IPEX phenotype.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

INCIDENCE, ONSET OF SYMPTOMS, AND AGE AT DIAGNOSIS

Because there is no broad-based screening program for IPEX, the true incidence of this disease is unknown. Following the discovery of the genetic defect in IPEX, more than 100 unrelated families have been identified, 52 in our laboratory. The triad of severe diarrhea associated with villous atrophy and failure to thrive, an eczematous dermatitis, and early-onset diabetes is highly suggestive of classic IPEX. As one would expect, there are milder, less characteristic phenotypes not readily recognized as IPEX. The oldest described case in which a de novo diagnosis of IPEX was considered and confirmed by mutation analysis is a 19-year-old (Tourangeau et al., 2011). A positive

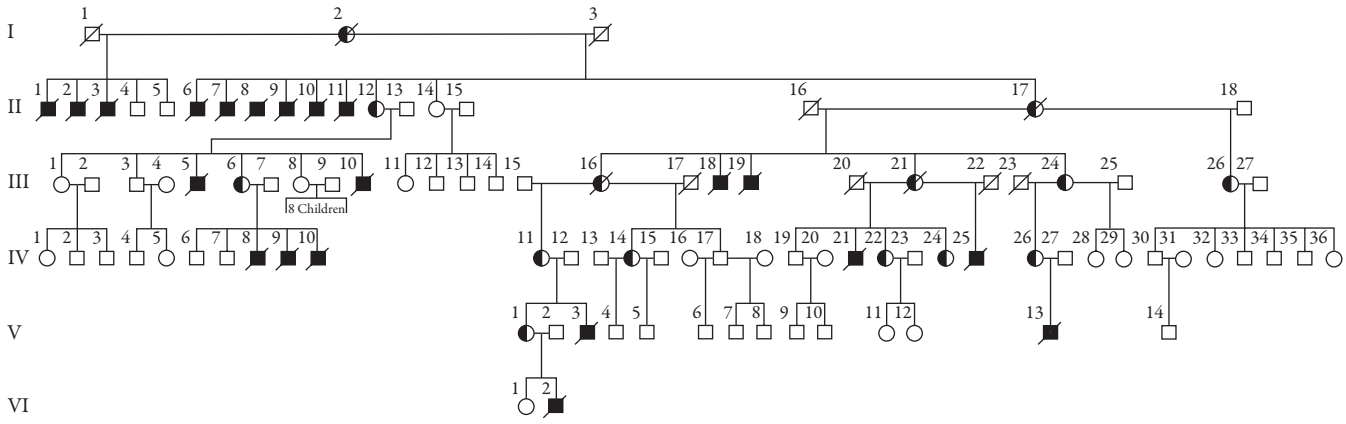

Figure 32.1 Updated pedigree of the IPEX kindred originally described by Powell et al. (1982), demonstrating X-linked inheritance and high mortality (half-filled circle, carrier female; filled square with slash, affected deceased male).

family history supports early recognition and prenatal diagnosis is possible if the *FOXP3* mutation is known for a given family.

GASTROINTESTINAL SYMPTOMS AND FAILURE TO THRIVE

Early-onset diarrhea associated with villous atrophy and lymphocytic infiltrates in the small bowel mucosa are the most prominent clinical findings in patients with the IPEX phenotype (An et al., 2011; Baud et al., 2001; Bennett et al., 2001b; Chatila et al., 2000; Cilio et al., 2000; Di Rocco and Marta, 1996; Ellis et al., 1982; Ferguson et al., 2000; Finel et al., 1996; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Harbuz et al., 2010; Hattevig et al., 1982; Jonas et al., 1991; Kobayashi et al., 1998; Levy-Lahad and Wildin 2001; McMurchy et al., 2010; Meyer et al., 1970; Moes et al., 2010; Moraes-Vasconcelos et al., 2008; Peake et al., 1996; Powell et al., 1982; Roberts and Searle, 1995; Rubio-Cabezas et al., 2009; Satake et al., 1993; Savage et al., 1982; Seidman et al., 1990; Torgerson et al., 2007; Walker-Smith et al., 1982; Wildin et al., 2002; Zeller et al., 1994). With two exceptions, all patients in our series of 52 families with IPEX or those with IPEX-like phenotype presented with gastrointestinal symptoms of watery or mucoid-bloody diarrhea that does not respond to dietary manipulation (Table 32.1). Total parenteral nutrition (TPN) is often required to prevent failure to thrive. Severe villous atrophy and mucosal erosion with lymphocytic infiltrates of the submucosa or lamina propria are frequently observed in small bowel biopsies, often leading to a diagnosis of autoimmune enteropathy and occasionally of Crohn's disease or ulcerative colitis. In rare cases, gastrointestinal symptoms occur at a later age, possibly resulting from a less severe mutation of *FOXP*3 or, in some cases, non-FOXP3-related mechanisms. Treatment with immunosuppressive drugs may lead to temporary improvement of gastrointestinal symptoms.

AUTOIMMUNE ENDOCRINOPATHY

Early-onset, sometimes neonatal or congenital insulin-dependent type 1 diabetes is a frequent initial finding (Bae et al., 2011; Bennett et al., 2001a; Cilio et al., 2000; Di Rocco and Marta, 1996; Dodge and Laurence, 1977; Ferguson et al., 2000; Finel et al., 1996; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Hattevig et al., 1982; Jonas et al., 1991; Levy-Lahad and Wildin 2001; Meyer et al., 1970; McMurchy et al., 2010; Moes et al., 2010; Moraes-Vasconcelos et al., 2008; Nieves et al., 2004; Peake et al., 1996; Powell et al., 1982; Roberts and Searle 1995; Satake et al., 1993; Wildin et al., 2002; Zeller et al., 1994). IPEX patients with diabetes lack sufficient insulin and often have anti–islet cell antibodies. At autopsy, chronic inflammation with lymphocytic infiltrates in the pancreas and absence of islet cells is a characteristic finding. Thyroid disease, initially presenting as either hypo- or hyperthyroidism, is a common complication (Gambineri et al., 2008; Halabi-Tawil et al., 2009; Moes et al., 2010; Moraes-Vasconcelos et al., 2008; Nieves et al., 2004; Ochs et al., 2007; Powell et al., 1982; Satake et al., 1993; Wildin et al., 2001, 2002) and may be associated with elevated thyrotropin levels or anti-thyroid microsomal antibodies (Kobayashi et al., 1998; Savage et al., 1982; Wildin et al., 2002).

In our own clinical survey of IPEX patients, type 1 diabetes was present in 55 percent and thyroid disease in 33 percent of patients with a *FOXP*3 mutation. Interestingly, only 1 of 52 IPEX patients had adrenal insufficiency.

AUTOIMMUNE HEMATOLOGICAL DISORDERS

Coombs-positive hemolytic anemia, autoimmune thrombocytopenia, or neutropenia are frequent complications (Baud et al., 2001; Di Rocco and Marta, 1996; Gambineri et al., 2008; Levy-Lahad and Wildin 2001; Nieves et al., 2004; Ochs et al., 2007; Powell et al., 1982; Roberts and Searle, 1995; Satake et al., 1993; Wildin et al., 2002). Specific autoantibodies (anti-red blood cell, antiplatelet, or antineutrophil antibodies) are frequently present in the circulation.

AUTOIMMUNE HEPATITIS

Hepatitis is a frequent complication observed in IPEX patients (Gambineri et al., 2008). In our own series, 22 percent of IPEX

Table 32.1 SINGLE-GENE DEFECTS CAUSING A PHENOTYPE OF IMMUNE DYSREGULATION

GENE (CHAPTER#)	*FOXP3 (32)*	*CD25*	*STAT5B (32)*	*FAS/FASL (30)*	*AIRE (31)*	*RAG1/RAG2; ARTEMIS (13)*	*ITCH/AIP4 (32)*	*STAT1 (32)*
Human Disease	IPEX	CD25 deficiency (32)	STAT5b deficiency	ALPS	APECED	Omenn Syndrome	ITCH deficiency	IPEX-like, heterozygous gain of function mutation
Mouse	Scurfy	CD25 knockout	STAT5a/5b double knockout	MRLlpr/glp	Aire-knock out	Rag1/2 knockout	itchy mouse	Not known
Onset of Symptoms								
Human	early infancy	infancy	variable, often early	childhood	4–5 years	infancy	childhood	childhood
Mouse	1st week of life	early	early	early	late (6–30 weeks)	late	early	–
Lethality								
Human	infancy	infancy	adult	adulthood	adulthood	infancy	early adulthood	adulthood
Mouse	3 weeks	late	early	late	late	–	late	
Clincal Feature								
Endocrinopathy	+ + + diabetes, thyroiditis	+ + + diabetes		(+)	+ + + + adrenal failure, hypoparathyroidism	–	+ + + diabetes, thyroiditis	+ + + diabetes, thyroiditis
Enteritis	+ + + +	+ + + +	+	(+)	(+)	+ + + +	+ + +	+ + + +
Skin lesions	+ + + + erythema, eczema, psoriasi form, alopecia	+ + + eczema	+ eczema	–	+ + + ectodermal dystrophy mucocutaneous candidiasis, vitiligo, alopecia	+ + + + enythroderma	+ + + itchy dermatitis	+ + + eczema
Hemolytic anemia Thrombocytopenia	+ + +	–	–	+ +	+	–	–	+/–
lymphadenopathy	+ +	+ + +	–	+ + + +	+ +	+ + +	–	+/–
hepatosplenomegaly	+ +	+ + +	–	+ + + +	+ (autoimmune hepatitis)	+ + +	+ + + (autoimmune hepatitis)	+ + + +
Gonadal failure	–	–	+	–	+ +	–	–	–
Renal disease	+ + +	–	–	(+)	–	–	–	–

(continued)

Table 32.1 (CONTINUED)

GENE (CHAPTER#)	*FOXP3 (32)*	*CD25*	*STAT5B (32)*	*FAS/FASL (30)*	*AIRE (31)*	*RAG1/RAG2; ARTEMIS (13)*	*ITCH/AIP4 (32)*	*STAT1 (32)*
Lab Abnormalities								
Serum immunoglobulins, T cells	elevated IgA, low CD4+CD25+FOXP3+	low IgG, IgA, IgM, immune deficient (SCID-like), lymphopenia	increased IgG, IgA, IgM, T cell lymphopenia, low CD4+CD25+FOXP3+ T cells, immune deficiency with growth hormone insensitivity	increased IgG, IgA, IgM1		low IgG, IgA, IgM low # of B cells and elevated number of T cells, decreased lymphocyte proliferation		mucocutaneous fungal infections, viral infections, decreased IL-17 producing cells, B and T cell lymphopenia in some, decreased class switched memory B cells, abnormal antibody responses
Elevated IgE	+ + +	–	–	+	–	+ + +	–	–
Eosinophilia	+ +	–	–	+	–	+ + +	–	–
Auto-antibodies	anti-β cells; anti-blood cells; anti-enterocytes	+		anti-blood cells	anti-parathyroid, anti-adrenal cortex anti-IL-17A, F anti-IL-22	–	anti-liver/kidney microsomal Ab, ANA, anti-neutrophil, anti-enterocyte Ab, anti-thyrogloblulin	–
Lymphocytic infiltrates	activated T cells in lymph nodes, spleen, liver, pancreas, skin	skin, lung, gut, liver	lungs, interstitial pneumonia	double negative α/β+ immature T cells; lymph nodes, spleen, liver	adrendal gland, gonades	skin, lymph nodes	pulmonary infiltrates	–

patients with mutations in FOXP3 have a history of hepatitis. In some patients, the liver disease is associated with the presence of high titers of anti-liver/kidney microsomal antibody (LKM1 type 2), or antimitochondrial antibody, autoantibodies associated with autoimmune hepatitis or primary biliary cirrhosis (PBC), respectively (Lopez et al., 2011; Tsuda et al., 2010).

DERMATOLOGICAL ABNORMALITIES

Dermatitis is one of the most consistent clinical findings in IPEX (Baud et al., 2001; Chatila et al., 2000; Di Rocco and Marta, 1996; Ellis et al., 1982; Ferguson et al., 2000; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Levy-Lahad and Wildin, 2001; McGinness et al., 2006; Moes et al., 2010; Moraes-Vasconcelos et al., 2008; Nieves et al., 2004; Peake et al., 1996; Powell et al., 1982; Roberts and Searle, 1995; Zeller et al., 1994). Eczema is by far the most common skin manifestation observed in patients of all ages with IPEX (Fig. 32.2 and Color Plate 32.I), but erythroderma has been reported in a few infants with the disease. In older patients, severe localized psoriasiform dermatitis (Fig. 32.3 and Color Plate 32.IIA), alopecia universalis, and pemphigoid nodularis have been described (McGinness et al., 2006; Nieves et al., 2004). The psoriasiform lesions show irregular hyperplasia of the epidermis with overlying parakeratosis and lymphocytic infiltrates (Fig. 32.4 and Color Plate 32.IIB). Autoantibodies recognizing skin antigens, including Keratin 14, have been identified in the blood of scurfy mice and IPEX patients (Huter et al., 2010). The usual eczematous dermatitis of IPEX is responsive to T-cell–directed immunosuppression (tacrolimus, etc.) and topical therapy with steroid ointment and hydration. Pemphigoid nodularis observed in a 14-year-old boy with autoantibodies to the 180 Kd bullous pemphigoid antigen was responsive to B-cell–depletion therapy with monoclonal anti-CD20 antibody (McGinness et al., 2006).

INFECTIONS

It is unclear whether IPEX patients have a significantly increased susceptibility to infections as a function of their genetic defect, as was suspected by Powell et al. (1982), or whether this susceptibility is secondary to other clinical features, such as decreased barrier function of the skin and gut, or to immunosuppressive therapy. In a large cohort of patients with the IPEX phenotype, 62 percent had serious infection, including sepsis, meningitis, pneumonia, and osteomyelitis, with many of these infections occurring prior to the initiation of immunosuppressive therapy (Ochs and Torgerson, 2007). Sepsis due to line infection is a common complication. Autoimmune neutropenia, if present, likely contributes to the increased incidence of infections in those patients with this feature. The most common pathogens identified were *Enterococcus* and *Staphylococcus* species, cytomegalovirus, and *Candida* (Ferguson et al., 2000; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Jonas et al., 1991; Kobayashi et al., 1998; Levy-Lahad and Wildin, 2001; Moraes-Vasconcelos et al., 2008; Ochs and Torgerson, 2007; Peake et al., 1996; Roberts and Searle, 1995). Opportunistic infections such as *Pneumocystis jiroveci* are rare and are most likely a consequence of immunosuppressive therapy (Torgerson et al., 2007).

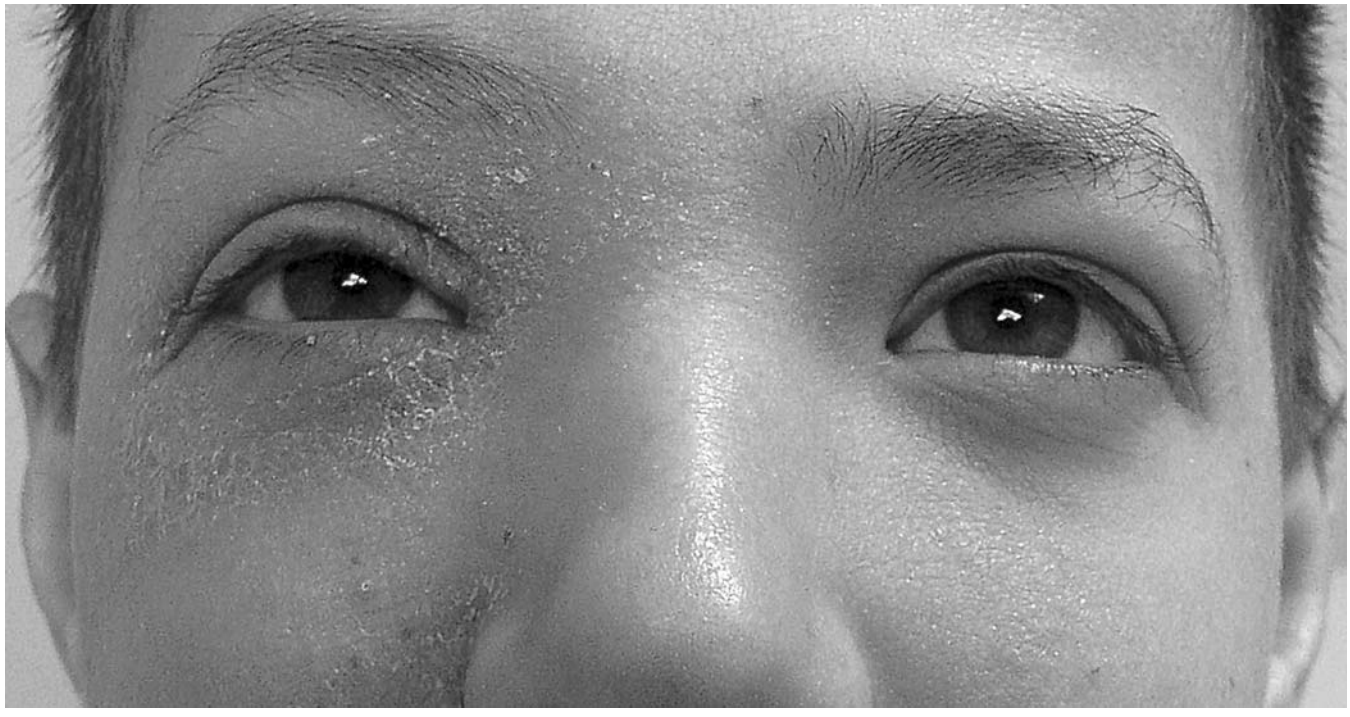

Figure 32.2 Chronic eczema in a 14-year-old male IPEX patient. (See Color Plate.)

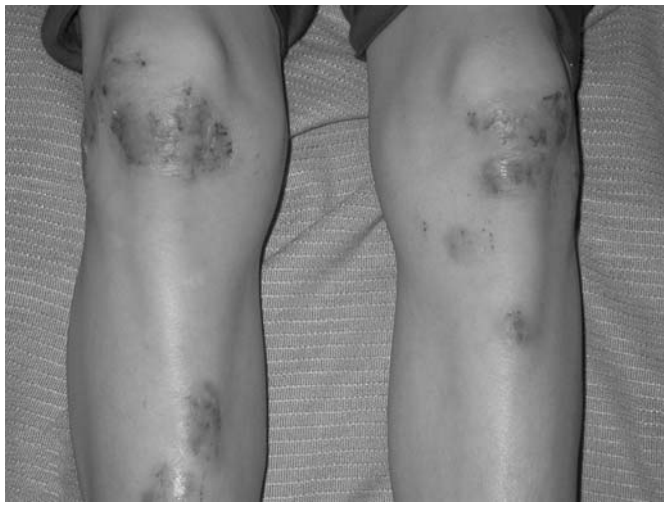

Figure 32.3 Psoriasiform dermatitis in a patient with IPEX due to an amino acid substitution in FOXP3 (p.A384T). Chronic scaly, erythematous plaques are present on the patient's legs. (See Color Plate.)

OTHER CLINICAL MANIFESTATIONS

Severe, acute reactions to routine immunizations have been reported (Powell et al., 1982). Such reactions suggest that FOXP3 and regulatory T cells are necessary to keep immune responses to foreign antigens under control and prevent the potential of "horror autotoxicus" or autoimmunity, a term introduced by Paul Ehrlich more than 100 years ago (Ehrlich, 1900).

Renal disease, described as glomerulonephropathy, interstitial nephritis (Bae et al., 2011; Ellis et al., 1982; Kobayashi et al., 1998; Powell et al., 1982; Zeller et al., 1994), or minimal-change nephritic syndrome (Hashimura et al., 2009; Rubio-Cabezas et al., 2009) has been observed frequently in IPEX patients. In our own IPEX series, 29 percent of patients with *FOXP*3 mutation had renal abnormalities. In some instances, renal disease may be directly caused or worsened by treatment with calcineurin inhibitors; however, renal disease has been described in patients not receiving any immunosuppressive drugs.

Other features include neurological problems, including seizures and developmental delay, in almost 25 percent of the patients in our cohort. In addition, hepatosplenomegaly and lymphadenopathy due to extensive lymphocytic infiltrates have been reported in patients at autopsy (Levy-Lahad and Wildin, 2001; Peake et al., 1996; Roberts and Searle, 1995; Wildin et al., 2002).

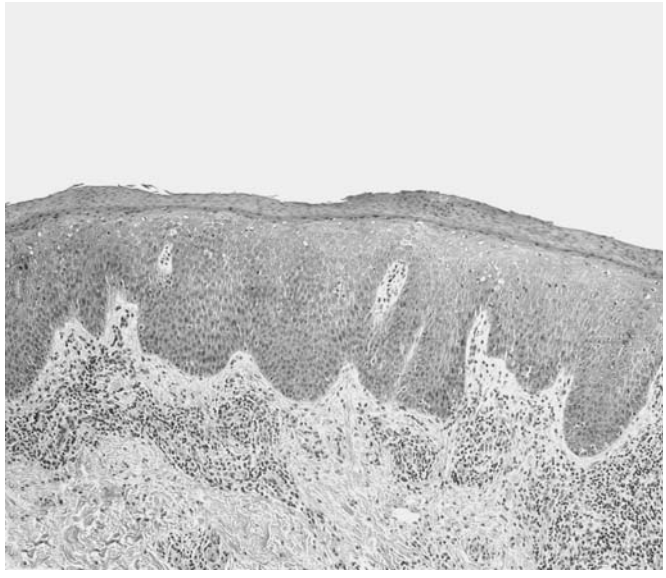

Figure 32.4 A skin biopsy of a new skin lesion of the same patient shows irregular psoriasiform hyperplasia of the epidermis with overlying parakeratosis. Cell infiltrates consist mainly of T lymphocytes (CD8>CD4) (hematoxylin-eosin, ×10). (From Nieves et al., *Arch Dermatol* 2004;140:466–472, with permission.) (See Color Plate.)

LABORATORY FINDINGS

The immunological evaluation of IPEX patients is unremarkable except for elevated serum levels of IgE and IgA and marked eosinophilia. IgG and IgM levels, absolute lymphocyte counts, and subset numbers are typically within the normal range, and in vitro proliferation of lymphocytes in response to mitogens and specific antigens is normal (Gambineri et al., 2008). In our series, greater than 90 percent of IPEX patients had significantly elevated IgE levels and 67 percent had elevated IgA levels.

With only rare exceptions, patients with IPEX have normal antibody responses to immunization with protein and polysaccharide antigens (for review, see Wildin et al., 2002). In those patients who do not respond, it may be a result of systemic immunosuppressive therapy at the time of immunization and testing. The presence of autoantibodies is a hallmark of this syndrome (Gambineri et al., 2008; Huter et al., 2010; Tsuda et al., 2010). Most patients with insulin-dependent diabetes have autoantibodies against pancreatic islet cells (Baud et al., 2001; Ferguson et al., 2000; Finel et al., 1996; Gambineri et al., 2008; Jonas et al., 1991; McMurchy et al., 2010; Peake et al., 1996; Roberts and Searle, 1995; Wildin et al., 2002). Anti-insulin and anti-glutamic acid decarboxylase (GAD) antibodies are also common (Baud et al., 2001; Cilio et al., 2000; Gambineri et al., 2008; McMurchy et al., 2010). In addition, antimicrosomal/antithyroglobulin antibodies, antibodies against smooth muscle, mitochondrial antigens, and antinuclear antibodies have been demonstrated (Baud et al., 2001; Di Rocco and Marta, 1996; Ferguson et al., 2000; Finel et al., 1996; Gambineri et al., 2008; Powell et al., 1982; Satake et al., 1993; Savage et al., 1982; Seidman et al., 1990; Tsuda et al., 2010). Antibodies against human intestinal tissue (anti-enterocyte) and an autoantibody specific for a 75kDa gut- and kidney-specific antigen (AIE-75) have been observed (Gambineri et al., 2008; Kobayashi et al., 1998, 1999; McMurchy et al., 2010; Satake et al., 1993; Walker-Smith et al., 1982). Studies of scurfy mice and patients with IPEX demonstrated the presence of autoantibodies that target keratins (Huter et al., 2010).

While $CD4^{+}CD25^{high}$ lymphocytes are present in the peripheral blood of IPEX patients with mutations in FOXP3, most, but not all, have low percentages of

$CD4^+CD25^{high}FOXP3^+$ Treg cells in the circulation (Gambineri et al., 2008; Gavin et al., 2006). It is unlikely that the $FOXP3^+$ cells that are present exhibit significant suppressive activity. Due to the volume of blood required to perform the routine in vitro suppression assays to evaluate Treg function and the fact that most patients are small and clinically unstable, this cannot be formally evaluated in most patients at the present time. Flow cytometry testing to evaluate Treg number and percentage is therefore the recommended screening test. CD4, CD25, and FOXP3 are generally considered to be the basic markers needed to identify Tregs by flow cytometry. This can however be further refined by using additional markers, including low-level expression of CD127 (the α-chain of the interleukin [IL]-7 receptor), CTLA-4, GITR, and Helios. A number of studies have demonstrated that CD127 expression on Tregs inversely correlates with FOXP3 expression and the suppressive function of Treg cells, making it a particularly useful cell-surface marker in combination with CD4 and CD25 for isolation of viable $CD4^+CD25^+CD127^{low}FOXP3^+$ Treg cells (Hartigan-O'Connor et al., 2007; Liu et al., 2006; Shen et al., 2009; Smith et al., 2008; Otsubo K, et al. 2011; Venken et al., 2008).

MOLECULAR BASIS OF IPEX

Following the identification of a 2bp insertion in the *Foxp*3 gene of mice with the scurfy phenotype (Brunkow et al., 2001), three laboratories reported that symptomatic males of families with X-linked IPEX had mutations of the human *FOXP*3 gene (Bennett et al., 2001b; Chatila et al., 2000; Wildin et al., 2001). The human *FOXP*3 gene, located on the short arm of the X chromosome (Xp11.23), consists of 11 translated exons that encode a protein of 431 amino acids (429 amino acids for the murine Foxp3). The two proteins, human and murine, have 86 percent sequence identity. The gene is expressed predominantly in lymphoid tissues (thymus, spleen, and lymph nodes), particularly in $CD4^+CD25^{bright}$ T cells (Roncador et al., 2005). Human $CD4^+CD25^-$ and $CD8^+$ T cells can inducibly express FOXP3 upon activation, although typically at lower levels than in Treg cells (Cosmi et al., 2003; Roncador et al., 2005; Walker et al., 2003; Xystrakis et al., 2004). In mice, Foxp3 is expressed at low levels in $CD4^+CD25^-$ but not at significant levels in CD8+ cells (Brunkow et al., 2001; Fontenot et al., 2005; Hori et al., 2003).

The Forkhead (FKH) BOX protein FOXP3 is a member of the P subfamily of Fox transcription factors, which as a group are characterized by the presence of a highly conserved winged helix/forkhead DNA binding domain. Proteins bearing a forkhead DNA-binding motif represent a large family of related transcriptional regulators that play diverse roles in enhancing or suppressing transcription of particular genes. Studies in model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* have demonstrated a pivotal role for Fox family transcription factors in embryonic patterning, development and organogenesis, and regulation of metabolism (Benayoun et al., 2011; Carlsson and Mahlapuu, 2002; Gajiwala and Burley, 2000; Jackson et al., 2010). A subset of Fox transcription factor family members has been shown to play a role in the development and maintenance of normal immune responses and thymic development (Foxn1, see Chapter 21), in lineage commitment (Foxp3; e.g., $CD4^+CD25^+$ regulatory T cells), and in the function of lymphocytes (Foxj1 and Foxo3).

The FOXP3 protein has a number of characteristic structural features, including an amino-terminal proline-rich domain required for the gene repression activity of FOXP3 (Lopes et al., 2006), a centrally located C2H2 zinc-finger and leucine zipper (both conserved structural motifs involved in protein–protein interaction and the latter involved in dimerization of FOXP3), and carboxy-terminal forkhead DNA-binding domain (Fig. 32.5). There is a putative nuclear localization signal at the C-terminal portion of the forkhead domain (Lopes et al., 2006). Human Tregs co-express equal amounts of two splice isoforms of FOXP3, one encoded by the full-length mRNA and the other lacking the sequence encoded by exon 2 (FOXP3Δ2). At present, the function of FOXP3Δ2 is not fully understood (Allan et al., 2005).

FUNCTION OF FOXP3

For transcription factors such as FOXP3 to modulate gene transcription, they need to be imported to the nucleus of the cell, where they typically interact with a number of binding partners to either upregulate or downregulate transcription from a number of gene promoters. FOXP3 is no exception. Studies using N-terminal green fluorescent protein (GFP)-tagged FOXP3 suggest that it constitutively traffics to the nucleus (Fig. 32.6; Lopes et al., 2006). Studies using a combination of gene expression analysis and genome-wide chromatin immunoprecipitation to evaluate promoter occupancy have demonstrated that FOXP3 binds to more than 700 promoters and can act either as a transcriptional repressor or as a transcriptional activator, depending on the promoter to which it is bound (Zheng et al., 2007). Recent mouse data have demonstrated that on DNA, Foxp3 interacts with a network of other transcription factors to mediate its effects (Fu et al., 2012; Rudra et al., 2012). Specifically, when bound to the IL-2 promoter, crystal structure analysis suggests that the FOXP3 forkhead domain forms a ternary complex with the DNA-binding domain of the transcription factor NFAT1 (Bandukwala et al., 2011). The unique feature of this structure is that FOXP3 forms a domain-swapped dimer that bridges two molecules of DNA. Some IPEX-associated mutations, including the F373A mutation observed in one patient with a severe IPEX phenotype (Bacchetta et al., 2006; Otsubo et al., 2011; Wildin et al., 2002), are located in this domain swap interface of FOXP3, disrupting dimer formation by the Forkhead domain without affecting DNA binding; this defect in dimer formation was associated with loss of suppressor function (Bandukwala et al., 2011).

FOXP3 ACTS AS A TRANSCRIPTIONAL REPRESSOR OF CYTOKINE PROMOTERS

Among the important genes regulated by Foxp3 are a number of cytokine genes, including those encoding IL-2, IL-4,

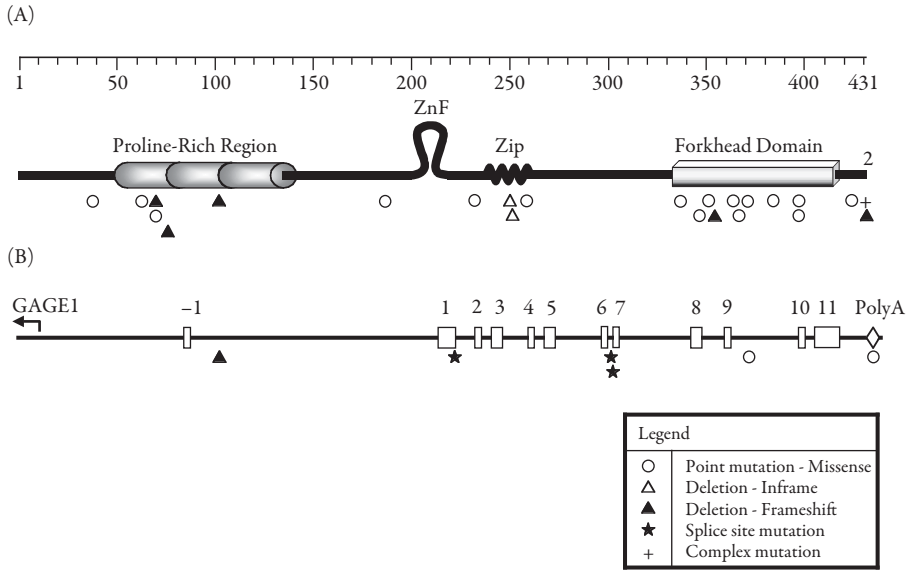

Figure 32.5 FOXP3 structure. (A) Schematic representation of the FOXP3 protein, highlighting the proline-rich region at the N terminus, followed by a C2H2 zinc-finger domain (ZnF), a leucine zipper (Zip), and the DNA-binding forkhead domain at the C terminus. (B) Genomic organization of the FOXP3 gene. There are 11 coding exons, a Poly A region located 878–883 bp downstream of the FOXP3 stop codon, and the adjacent (upstream) gene (*GAGE1*). The location and type of FOXP3 mutations identified to date are indicated by specific symbols. Nucleotide 1 is the first nucleotide of exon 1.

and interferon-γ (Bettelli et al., 2005; Schubert et al., 2001). Downregulation of gene transcription by Foxp3 on these promoters is thought to be the reason that Treg cells do not express these cytokines upon activation. Foxp3, but not Foxp1 or Foxp2, physically associates and functionally interacts with NF-κB and NFAT protein, and as a result inhibits IL-2, IL-4, and IFN-γ production by Foxp3-transduced $CD4^+$ effector cells. T cells from scurfy mice have a substantial increase in NFAT and NF-κB transcriptional activity compared with T cells derived from wild-type mice. This overexpression of NFAT and NF-κB is reduced to normal levels by complementation with Foxp3 in scurfy-derived T cells (Bettelli et al., 2005).

In addition to direct interactions on cytokine promoters, FOXP3 has been proposed to have a second effect caused by binding to and repressing transcription of the gene encoding the inducible form of NFAT (NFAT2) in T cells. In this way, FOXP3 functions not only to suppress the first wave of NFAT-mediated transcriptional responses mediated by NFAT1 as a result of direct interactions on cytokine promoters but also affects sustained NFAT-mediated inflammatory gene expression through suppression of inducible NFAT2 transcription (Torgerson et al., 2009). In addition, FOXP3 binds to intergenic regions, suggesting that FOXP3 also regulates noncoding RNA, such as miR-155, a miRNA associated with c-myc upregulation, pre-B-cell proliferation, and tumorigenesis (Zheng et al., 2007).

FOXP3 AS A RHEOSTAT OF THE IMMUNE RESPONSE

The generation of transgenic mice expressing multiple copies of the *Foxp*3 gene results in a dramatic suppression of immune responses. Transgenic mice have markedly decreased numbers of $CD4^+$ T cells in the peripheral blood and decreased cellularity in lymph nodes and spleen. In contrast to scurfy T cells, those derived from Foxp3 transgenic mice were hyporesponsive

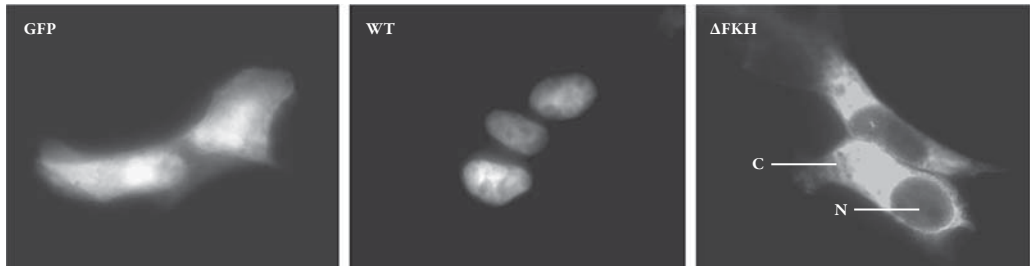

Figure 32.6 Nuclear import of FOXP3. The forkhead domain of FOXP3 is required for nuclear import. Constructs encoding N-terminal green florescent protein (GFP), GFP-FOXP3 (WT), or GFP-FOXP3 lacking the forkhead (ΔFKH) domain were transiently transfected into HEK 293 cells and evaluated by standard fluorescence microscopy for the ability to translocate to the nucleus. GFP by itself is below the size-exclusion limit for the nuclear pore complex and therefore is present throughout the cell. WT FOXP3 is located entirely in the nucleus; if the forkhead domain is deleted (ΔFKH-FOXP3), the protein is excluded from the nucleus (N) and remains in the cytoplasm (C).

to stimulation both in vivo and in vitro. The suppressive effect was shown to be entirely dependent on peripheral T cells, as overexpression of wild-type Foxp3 in the thymus did not affect peripheral-blood T-cell numbers or functions (Khattri et al., 2001). In this model, Foxp3 functions as a rheostat of the immune system, with activation responses being inversely proportional to the amount of Foxp3 protein expressed by $CD4^+$ T cells. Combined with the findings reported by Bettelli et al. (2005, vide supra), this hypothesis postulates that Foxp3 has an important function in peripheral $CD4^+$ T cells, in addition to its role in generating regulatory T cells in the thymus. If, as suggested by experimental evidence (Bettelli et al., 2005; Schubert et al., 2001; Torgerson et al., 2009), Foxp3 directly interacts with NF-κB and NFAT, it is possible that this physical association recruits Foxp3 to the promoters of cytokine genes. Being a potent transcriptional repressor, the recruitment of Foxp3 to these promoters would downmodulate transcriptional activity. Such a mechanism would explain the fact that scurfy T cells are hyperresponsive to T-cell receptor (TCR) stimulation with anti-CD3 (Clark et al., 1999). The observation that myelin proteolipid protein-specific autoreactive T cells transduced with Foxp3 are no longer able to mediate experimental autoimmune encephalomyelitis supports the notion that Foxp3 suppresses the effector function of autoreactive T cells (Bettelli et al., 2005). A similar suppression of experimental allergic encephalomyelitis by Foxp3-positive regulatory T cells was reported by Yu et al. (2005). Using a hemophilia A mouse model in which all T cells overexpressed FOXP3 (HemA/Foxp3-Tg), the transfer of factor VIII (FVIII) plasmid no longer induced the production of neutralizing anti-FVIII antibody in these HemA/Foxp3-Tg mice, resulting in permanent cure of hemophilia A (Miao et al., 2009). $CD4^+Foxp3^+$ T cells from these tolerized mice suppressed proliferation of FVIII-stimulated effector cells and could be adoptively transferred into plasmid-treated HemA mice, significantly suppressing the production of neutralizing antibody titers.

$CD4^+CD25^+FOXP3^+$ REGULATORY T CELLS

Some 10 years ago, Sakaguchi et al. described a small subset of $CD4^+$ T cells expressing the high-affinity IL-2 receptor α-chain (CD25) that was associated with antigen-specific suppression of T-cell responses (Sakaguchi et al., 2001). These $CD4^+CD25^+$ "regulatory" T cells (Tregs) are anergic in vitro but upon activation suppress proliferation and IL-2 production by naïve and memory $CD4^+$ T cells through a contact-dependent, cytokine-independent mechanism (Itoh et al., 1999; Shevach 2001). This type of "dominant tolerance" exerted by dedicated regulatory T cells is in contrast to the "recessive tolerance" mediated by negative selection of autoreactive T-cell clones in the thymus or autoreactive B-cell clones in the bone marrow or the periphery (see Chapters 30 and 31).

Regulatory T cells have been recognized as lead actors in the control of chronic human diseases. They play a major role in transplantation tolerance (Wood and Sakaguchi, 2003) and are numerically low in patients with chronic graft-versus-host disease following bone marrow transplantation (Zorn et al., 2005). Similarly, decreased numbers of regulatory T cells have been associated with autoimmune diseases in both humans and mice (Loser et al., 2005; Marinaki et al., 2005; Wei et al., 2004; Yu et al., 2005). Treatment strategies to increase Treg function have improved transplantation tolerance and autoimmune symptoms in experimental models (Bettelli et al., 2005; Liu et al., 2011; Loser et al., 2005). Conversely, an increase in the number and activation of Tregs has been associated with tumor progression (Berger et al., 2005; Karube et al., 2004; Ormandy et al., 2005; Unitt et al., 2005; Viguier et al., 2004), and therapeutic regimens are being explored to reduce Treg activity during cancer therapy (Beyer et al., 2005; Sharma et al., 2005; Wei et al., 2004).

Although the precise molecular events that lead to the production and regulation of regulatory T cells are unknown, it was shown independently by three groups that Foxp3 plays a crucial role in the generation of Tregs in mice (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Foxp3 is preferentially expressed in the $CD4^+CD25^+$ pool of cells, and mice that lack Foxp3 (scurfy or Foxp3 knockout mice) fail to generate functional Tregs. Ectopic expression of FOXP3 in human or murine $CD4^+CD25^-$ T cells is sufficient to convert these cells to a Treg-like phenotype regardless of CD25 expression, enabling the transduced T cells to suppress the proliferation of bystander cells in vitro and limit the effector functions of autoreactive T cells in vivo (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Yagi et al., 2004). Murine $CD4^+CD25^-$ cells, if activated in vitro by cross-linking CD3, do not express Foxp3 and do not acquire suppressor activity, despite strong expression of CD25 on the cell surface (Fontenot et al., 2003; Khattri et al., 2003). However, human $CD4^+CD25^-$ cells readily induce expression of FOXP3 when activated in vitro and develop some regulatory function, although not as potently as natural Treg cells (Walker et al., 2003; Yagi et al., 2004).

Although it is generally accepted that Foxp3 is indispensable for the differentiation and suppressive function of Tregs, it is unclear how $CD4^+CD25^+FOXP3^+$ regulatory T cells exert their potent suppressive effect on target cells to control immune responses to infectious agents and self-antigens, thus preventing autoimmunity. Most IPEX patients with known FOXP3 mutations (even those with missense mutations that allow expression of a mutant protein) lack a normal population of FOXP3-expressing $CD4^+CD25^+$ Tregs (Fig 32.7 and Color Plate 32.III) (Fuchizawa et al., 2007; Gavin et al., 2006). There are, however, IPEX patients with mutations in FOXP3 who have normal percentages of FOXP3+ T cells, but with impaired suppressive function (Bacchetta et al., 2006; Bandukwala et al., 2011; Gambineri et al., 2008).

Although the Treg story is still in flux, several key facts are generally accepted: Naturally arising $CD4^+CD25^+FOXP3^+$ Tregs are generated in the thymus of both mice and humans (Itoh et al., 1999; Papiernik et al., 1998; Stephens and Mason 2000; Roncador et al., 2005). These $CD4^+CD25^+FOXP3^+$ thymocytes are capable of suppression in adoptive transfer models and in in vitro suppression assays (Itoh et al., 1999). Furthermore, the generation of Tregs in the thymus requires TCR signaling during thymocyte development (Hsieh and Rudensky, 2005). In this model of regulatory T-cell lineage commitment, intermediate-avidity TCR–self-peptide–MHC interactions take

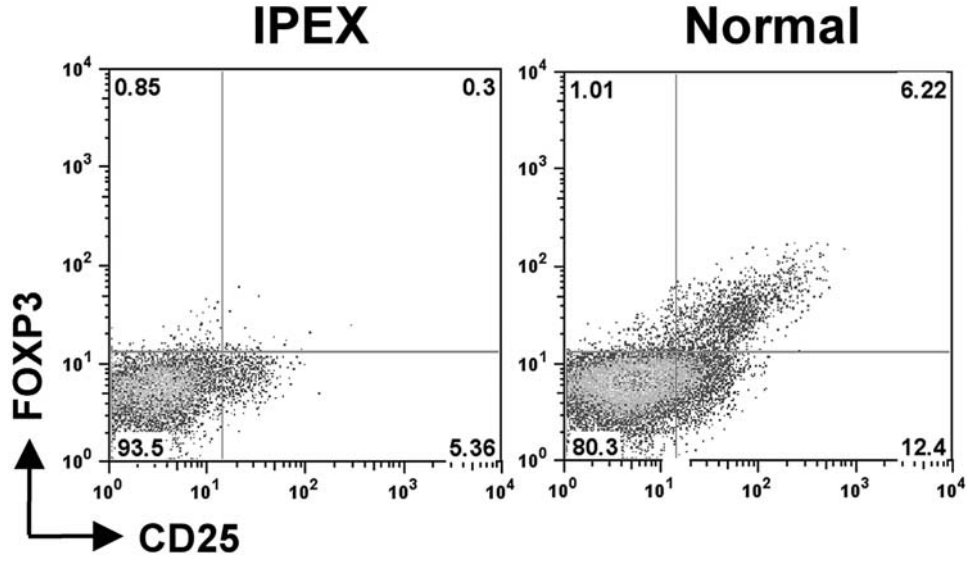

Figure 32.7 Regulatory T cells (Tregs) in human peripheral blood. Peripheral blood mononuclear cells were stained for CD4, CD25, and FOXP3 using fluorescently labeled monoclonal antibodies and evaluated by flow cytometry. A subset of CD4+ T cells (5–7 percent in normal individuals) are $CD4^+CD25^{bright}FOXP3^+$ and constitute the naturally arising regulatory T-cell population. This subset of $CD4^+CD25^{bright}FOXP3^+$ Treg cells is absent in the peripheral blood of a patient with IPEX. (See Color Plate.)

place in the thymus, augmented by CD28 signaling and signals sent via common γ-chain-containing cytokine receptors (and perhaps additional, unidentified signals) to induce Foxp3 expression and Treg cell differentiation. While Foxp3 is crucial for development of Treg cells in the murine thymus, its continuous expression is equally important for the maintenance of Treg cell function. Induced excision of a conditional (loxP-flanked) Foxp3 allele in mature peripheral murine Treg cells resulted in the loss of Foxp3 protein and ablation of suppressor function while gaining the ability to produce IL-2, IFN-γ, and TNF (Williams and Rudensky, 2007). Similar results were observed when wild-type mice harboring Treg cells that could be deleted at will were generated by inserting cDNA encoding GFP fused to the diphtheria toxin receptor into the 3′ UTR of the Foxp3 locus. Since all Foxp3 Treg cells of these mice express the diphtheria toxin receptor, diphtheria toxin treatment results in rapid and complete elimination of Treg cells in these unmanipulated disease-free mice. Following chronic ablation of Treg cells, both neonatal and adult mice developed rapidly progressing terminal autoimmune disease and died within 10 to 24 days after diphtheria toxin exposure (Kim et al., 2007). In addition to controlling T-cell self-reactivity, an important role for Tregs in maintaining human B-cell tolerance has recently been demonstrated (Kinnunen et al., 2013). While new emigrant/transitional B cells from IPEX patients produced antibodies with reactivities similar to those generated by the same cell populations obtained from normal controls, mature "naïve" B cells from IPEX patients often expressed autoreactive antibodies. These studies demonstrate that Tregs represent a dedicated T-cell lineage capable of controlling both T- and B-cell self-reactivity, thereby preventing autoimmune disorders. The role of Foxp3, therefore, is that of being the most important lineage specification factor for Treg cells (Fontenot and Rudensky, 2005).

MUTATION ANALYSIS

In an effort to define the clinical and immunological phenotype of IPEX and to explore a possible genotype–phenotype correlation, we have identified FOX3 mutations in 70 patients from 52 families living in North or South America, Europe, or Asia with a clinical phenotype compatible with IPEX. To date, we have identified 32 unique FOXP3 mutations (Fig. 32.5), in addition to mutations reported previously by others (An et al., 2011; Bacchetta et al., 2006; Bae et al., 2011; Chatila et al., 2000; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Harbuz et al., 2010; McMurchy et al., 2010; Moes et al., 2010; Otsubo et al., 2011; Owen et al., 2003; Rubio-Cabezas et al., 2009; Tsuda et al., 2010; Wildin et al., 2001, 2002). These naturally occurring mutations have been invaluable for understanding the relative importance of each structural domain of FOXP3. The missense mutations identified thus far cluster in three specific functional domains: the N-terminal proline-rich domain, the central leucine zipper, and the C-terminal forkhead domain (Fig. 32.5). No missense mutations have been discovered in the zinc-finger domain, which suggests that it may not play a critical role in FOXP3 function. Other mutations identified include nonsense mutations, deletions, and splice-site mutations. In one family with multiple affected members, we found a large deletion upstream of exon 1 resulting in failure to initiate splicing (Moes et al., 2010). The family initially described by Powell in 1982 has a point mutation affecting the first canonical polyadenylation signal (AAUAAA→AAUGAA), which resides 878–883 bp downstream of the *FOXP*3 stop codon (Bennett et al., 2001a). This mutation may result in a somewhat milder phenotype, permissive of longer survival in some patients. However, except for one successfully transplanted infant, all affected members of that family eventually succumbed to this disease (Fig. 32.1). Not all patients who present with the IPEX phenotype have detectable

mutations in FOXP3. Of more than 400 patients with symptoms compatible with IPEX referred to our laboratory for sequencing, only 70 had identifiable mutations in *FOXP*3. Among the patients who had IPEX symptoms but lacked FOXP3 mutations (termed IPEX-like), approximately half were found to have low FOXP3 expression levels based on flow cytometry and quantitative polymerase chain reaction (PCR) testing. This suggests that at least some IPEX-like patients may have mutations involving regulatory sequences of the *FOXP*3 gene such as promoter or enhancer regions. Alternatively, there may be other genes or gene products that are directly or indirectly involved in the activation of FOXP3 itself.

STRATEGIES FOR DIAGNOSIS

Because of the wide spectrum of clinical findings, the diagnosis of IPEX should be considered in male patients presenting with intractable diarrhea, villous atrophy, and failure to thrive. The presence of an erythematous/eczematoid rash or a psoriasiform dermatitis supports the diagnosis (Figs. 32.2 and 32.3, Color Plates 32.I and 32.IIA). Early-onset type 1 insulin-dependent diabetes and/or hypothyroidism in a patient with gastrointestinal symptoms and eczema is almost pathognomonic for IPEX. Autoimmune hemolytic anemia, thrombocytopenia, and neutropenia are quite common. The diagnosis of IPEX is strongly suggested by demonstrating the absence of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Fig. 32.7 and Color Plate 32.III) (Bacchetta et al., 2006; Fuchizawa et al., 2007; Gambineri et al., 2008; Gavin et al., 2006; Otsubo et al., 2011) and confirmed by mutation analysis of *FOXP*3.

DIFFERENTIAL DIAGNOSIS

Other single-gene defects in either humans or mice found to cause clinical phenotypes with significant immune dysregulation include the following (Table 32.1):

CD25 deficiency. CD25 (α-chain of the IL-2 receptor) was found to be deficient in three infants from two unrelated families. Clinical features resemble those of both IPEX and SCID. Like IPEX, CD25-deficient infants presented with severe, chronic diarrhea and villous atrophy at an early age (Caudy et al., 2007; Sharfe et al., 1997). One patient presented with early-onset insulin-dependent diabetes and eczema (Caudy et al., 2007), and all subsequently developed autoantibodies, hepatosplenomegaly, lymphadenopathy, and lymphocytic infiltrates in the lung, gut, and liver (Aoki et al., 2006; Caudy et al., 2007; Sharfe et al., 1997). In addition to autoimmune symptoms, the patients had infectious complications more commonly observed in patients with SCID, including recurrent cytomegaloviral pneumonitis, persistent thrush, candidal esophagitis, and Epstein-Barr virus infection. One patient underwent successful bone marrow transplantation from a matched sibling (Aoki et al., 2006). CD25-deficient mice develop early autoimmunity with massive lymphocytic infiltrates in lungs and liver. Tregs from these mice have decreased competitive fitness relative to Tregs from normal mice (Boone et al., 2003; Sharma et al., 2009).

STAT5b deficiency. Signal transducer and activator of transcription 5b (STAT5b) is the transcription factor that mediates signaling from the IL-2, IL-7, and IL-15 receptors. As such, patients with STAT5b deficiency have moderate T- and NK-cell lymphopenia and are susceptible to chronic viral infections. Patients also have markedly decreased $CD4^+CD25^+FOXP3^+$ T cells and present with diarrhea, chronic lymphocytic interstitial pneumonitis (LIP), eczematous rash, and autoimmunity (Kofoed et al., 2003). Autoantibodies are present in half of the patients (Nadeau et al., 2011). In addition to cytokine signaling, STAT5b also plays an essential role in signaling from the growth factor receptor, so patients typically suffer from severe growth failure due to lack of IGF1 (insulin-like growth factor 1) that is not responsive to growth hormone therapy (Nadeau et al., 2011). STAT5a/5b double-deficient mice develop lymphocytic infiltrates of the bone marrow, colon, liver, and kidneys, resulting in early death. These mice have a quantitative reduction of Treg cells (Snow et al., 2003).

FAS/APO-1 (CD95) deficiency. FAS/APO-1 is a transmembrane receptor belonging to the tumor necrosis factor (TNF) receptor superfamily. Engagement of FAS/APO-1 by its ligand, FASL, induces programmed cell death in a variety of cell lines, including lymphocytes (Nagata 1997). Programmed cell death is an important mechanism of regulating the immune response by deleting autoreactive lymphocyte clones in the thymus and from the peripheral lymphocyte pool. Mutations in FAS or FASL cause a rare autosomal dominant syndrome known as autoimmune lymphoproliferative syndrome (ALPS) or Canale-Smith syndrome, which presents in the first 1 to 2 years of life and is characterized by massive lymphadenopathy and splenomegaly caused by nonmalignant lymphocytic infiltrates with an expansion of double-negative ($CD4^-CD8^-$) $\alpha\beta CD3^+$ T cells. Almost all patients present with autoimmune hemolytic anemia and thrombocytopenia, and some may have other autoimmune phenomena (Drappa et al., 1996; Fisher et al., 1995; ; Rieux-Laucat et al., 1995; see Chapter 30). Patients with ALPS survive well into adulthood and, as they age, continue to have autoimmune manifestations. Although the lymphoproliferative component of the syndrome often resolves with age, ALPS patients have a high incidence of B-cell lymphoma. Mice with inactivating mutations in either Fas or FasL show dramatic lymphocytic proliferation and infiltration of multiple organs. Fas/FasL mutant mice develop autoantibodies, including IgM rheumatoid factor and antichromatin antibodies (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Weintraub et al., 1998).

AIRE1 deficiency. Autoimmune regulator (AIRE) is a DNA-binding protein encoded by the *AIRE* gene located on chromosome 21 (Bjorses et al., 1998). AIRE is expressed in lymph nodes, spleen, testis, and thymus, particularly in the thymic medullary epithelial cells of the murine thymus (Heino et al., 2000). Mutations in this gene cause the rare autosomal recessive human syndrome of autoimmune polyendocrinopathy, chronic mucocutaneous candidiasis (CMC), ectodermal dysplasia (APECED) (see Chapter 31). The three most common characteristics of this disease are mucocutaneous

candidiasis, hypoparathyroidism, and adrenocortical insufficiency. Patients may develop a variety of other symptoms, including type 1 diabetes, Hashimoto's thyroiditis, vitiligo, and hemolytic anemia. The onset of APECED is less fulminant than that of IPEX, typically starting with candidiasis by age 5, hypoparathyroidism by age 10, and Addison's disease by age 15 (Vogel et al., 2002). Most APECED patients with CMC have high titers of neutralizing autoantibodies to IL-17A, IL-17F, and/or IL-22 (Kisand et al., 2010; Puel et al., 2010), strongly suggesting that the immune deficiency underlying CMC in APECED patients is caused by an autoimmune process. *Aire*-deficient mice, generated by targeted gene disruption, develop many of the typical APECED features (Anderson et al., 2002; Ramsey et al., 2002). Affected mice are born and develop normally but have lymphocytic infiltrates in the liver and atrophy of the adrenals and gonadal tissue. *Aire*-deficient mice often develop autoantibodies to the liver, exocrine pancreas, testis, and adrenal glands. Analysis of these deficient mice revealed that Aire is required for the expression of self-antigens by antigen-presenting thymic dendritic cells (Anderson et al., 2002). Following presentation of self-antigens to autoreactive T-cell clones, they are eliminated by negative selection (Su and Anderson, 2004). Thus, Aire provides a mechanism for the deletion of functional autoreactive T cells in the thymus, contributing substantially to immune tolerance.

Omenn syndrome. Omenn syndrome is a clinically defined disorder characterized by early-onset, generalized erythematous rash, combined immune deficiency, elevated serum IgE levels, and eosinophilia (see Chapter 13). Most patients with Omenn syndrome have normal numbers of (oligoclonal) T cells and low or absent circulating B-cell numbers. Because of severe gastrointestinal symptoms and failure to thrive, TPN is often required and line infection is a frequent complication. Hypomorphic mutations of *Rag*1 and *Rag*2 are frequently associated with Omenn syndrome, but mutations in Artemis, adenosine deaminase, common γ chain, RMRP, DNA-Ligase IV, or IL-7Rα have been reported in association with this clinical phenotype.

WAS deficiency. Patients with the Wiskott-Aldrich syndrome (WAS), caused by mutations in the *WAS* gene, frequently present during early infancy with bloody diarrhea, bacterial and viral infections, eczema, and bleeding due to congenital thrombocytopenia (see Chapter 43). Patients with WAS may develop autoimmune hemolytic anemia or thrombocytopenia, and neutropenia. Circulating CD4+CD25+FOXP3+ Tregs are normal in number but have impaired suppressive function. As in IPEX, the inheritance is X-linked.

ITCH deficiency. Mutations in the E3 ubiquitin ligase *ITCH/AIP4* have recently been observed in a large Amish kindred with 10 affected family members (Lohr et al., 2010). The clinical features include dysmorphic facial features, failure to thrive, developmental delay, and autoimmunity/immune dysregulation that is characterized by chronic interstitial pneumonitis (LIP), thyroiditis, type 1 diabetes, enteropathy, and hepatitis. Itch catalyzes the transfer of ubiquitin to a number of signaling proteins (PLCγ1, protein kinase Cθ, notch), a process that can lead to altered function or degradation of these proteins. Absence of Itch can affect T-cell anergy (Venuprasad 2010) or result in defective FOXP3 expression (Venuprasad et al., 2008). A radiation-induced loss-of-function mutation at the mouse agouti locus results in the non-agouti-lethal 18H mouse, also known as the itchy mouse (Perry et al., 1998). These Itch-deficient mice die at 4 to 6 months of age of chronic interstitial pneumonitis, gastroenteritis, lymphoid hyperplasia, and epidermal inflammation. Immunologically they demonstrate marked Th2 skewing within the T-cell population.

STAT1 Gain-of-Function Mutations. Heterozygous mutations in the transcriptional regulator Signal Transducer and Activator of Transcription 1 (STAT1) were recently identified in patients with chronic mucocutaneous candidiasis (CMC) (van de Veerdonk et al., 2011; Liu et al., 2011). These mutations within the coiled-coil and DNA-binding domains of the protein lead to hyperphosphorylation of STAT1 in response to cytokines such as IFNγ. Subsequent screening of a cohort of patients with IPEX-like symptoms who also had mucocutaneous fungal infections, found STAT1 gain-of-function mutations in a subset of these patients as well. Their clinical characteristics included enteropathy with villus atrophy, Type I diabetes (60%), thyroiditis (60%), mild-moderate eczema (100%), and a variety of other features including short stature (60%), delayed puberty, vascular aneurysms (20%), and viral infections. Interestingly, FOXP3+TREG numbers are within the normal range and have normal suppressive function as measured by in vitro assays (Uzel et al., 2013). The mechanism by which STAT1 gain-of-function patients develop severe autoimmunity is therefore unknown although it has been hypothesized that effector cells may be less responsive to suppression as a result of excessive STAT1 activity.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

If the mutation in a given family is known, a suspected carrier female can be identified by mutation analysis. Similarly, prenatal diagnosis of a male fetus at risk can be determined by DNA analysis with chorionic villus biopsy or cultured amniocytes as a DNA source.

TREATMENT AND PROGNOSIS

Early, aggressive therapy is of utmost importance in treating IPEX patients. By the time most patients are diagnosed, they typically demonstrate profound failure to thrive and malnutrition. They may also have diabetes and thyroid disease. The initial goal of therapy is to stabilize the patient in preparation for more definitive therapy. TPN is often necessary to ensure adequate caloric intake. This may require placement of a central venous catheter, which increases risks for line infection and sepsis. It is unclear if early immunosuppressive therapy can prevent the onset of type 1 diabetes. Long-term immunosuppression has been moderately effective in some patients, but over time, the effectiveness tends to diminish. Cyclosporin A or tacrolimus (FK506), sometimes in combination with steroids, is the most commonly used regimen (Baud et al.,

2001; Di Rocco and Marta, 1996; Ferguson et al., 2000; Finel et al., 1996; Kobayashi et al., 1995; Levy-Lahad and Wildin 2001; Satake et al., 1993; Seidman et al., 1990; Wildin et al., 2002). Sirolimus (rapamycin) has also been used with some success and seems to be less nephrotoxic (Bindl et al., 2005). Rituximab has been effective in situations where there is clearly autoantibody-mediated disease (pemphigoid nodularis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, etc.; McGinness et al., 2006). A variety of immunosuppressive medications, including methotrexate, corticosteroids, mycophenolate mofetil, azathioprine, infliximab, and others, have been tried but with mixed success. Chronic immunosuppressive therapy may facilitate opportunistic infections.

Hematopoietic stem cell transplantation is currently the only effective cure for IPEX, and many patients have achieved complete remission of symptoms following myeloablative (Baud et al., 2001; Mazzolari et al., 2005) or nonmyeloablative hematopoietic stem cell transplantation (Burroughs et al., 2010; Dorsey et al., 2009; Kasow et al., 2011; Rao et al., 2007; Seidel et al., 2009; Zhan et al., 2008). While many transplanted patients have achieved complete donor engraftment, several hematopoietic stem cell recipients had reduced donor T-cell engraftment ranging from 70 percent to as low as 5 to 10 percent (Baud et al., 2001; Mazzolari et al., 2005; Seidel et al., 2009). In the latter patient, despite total T-cell donor chimerism ranging from 5 to 10 percent, donor chimerism of the Treg cell population ranged between 53 and 91 percent. Importantly, this demonstrates that Tregs have a tremendous selective growth advantage in vivo and that long-term remission can be achieved with a minimal donor population of $CD4^+CD25^+CD127^{low}FOXP3^+$ Treg cells (Seidel et al., 2009). However, the overall long-term outcome of transplantation for IPEX is difficult to estimate since the number of reported transplants is relatively small and some transplanted patients have died of late complications after transplant (Baud et al., 2001; Wildin et al., 2002). The degree of symptomatic remission following stem cell transplantation depends on the transplant being initiated prior to irreversible damage that may have occurred to target organs such as the pancreas and thyroid. Interestingly, the patient reported by Baud et al. had complete symptomatic remission, including reversion of type 1 diabetes, during the conditioning regimen that consisted of anti–T-lymphocyte globulin, busulfan, and cyclophosphamide. This result raises the possibility that cytotoxic or biological agents that target T cells may be an effective treatment in patients who have failed to respond to other therapies. In general, the prognosis of IPEX is poor without stem cell transplantation. Untreated, most patients die at an early age. Patients with certain point mutations or unusual mutations, such as those affecting the mRNA polyadenylation signal, may present with a somewhat milder initial clinical course, but one that still leads to premature death. Treatment with potent immunosuppressive agents that target T cells can make a difference in these patients, but because of toxicity and increased susceptibility to infection, these agents do not guarantee long-term amelioration of symptoms. Hematopoietic stem cell transplantation should be performed early in the course of the disease, before pancreatic islet cells and other target organs have been irreversibly damaged.

ANIMAL MODELS

THE *SCURFY* MUTATION, A MOUSE MODEL FOR IPEX

The *Scurfy (sf)* mutation arose spontaneously in a partially inbred strain of mice at the Oakridge National Laboratory (Russell et al., 1959). Shortly after birth, affected male mice develop a scaly rash most evident on the ears and tail (Fig. 32.8 and Color Plate 32.IV), and they develop severe runting secondary to chronic diarrhea and malabsorption. Characteristically, the affected males also exhibit lymphadenopathy, splenomegaly, and massive lymphocytic infiltrates in the skin, liver, and lungs and develop hemolytic anemia associated with a positive Coombs test, suggesting that the *sf* mutation causes a "generalized autoimmune-like syndrome" (Godfrey et al., 1991b).

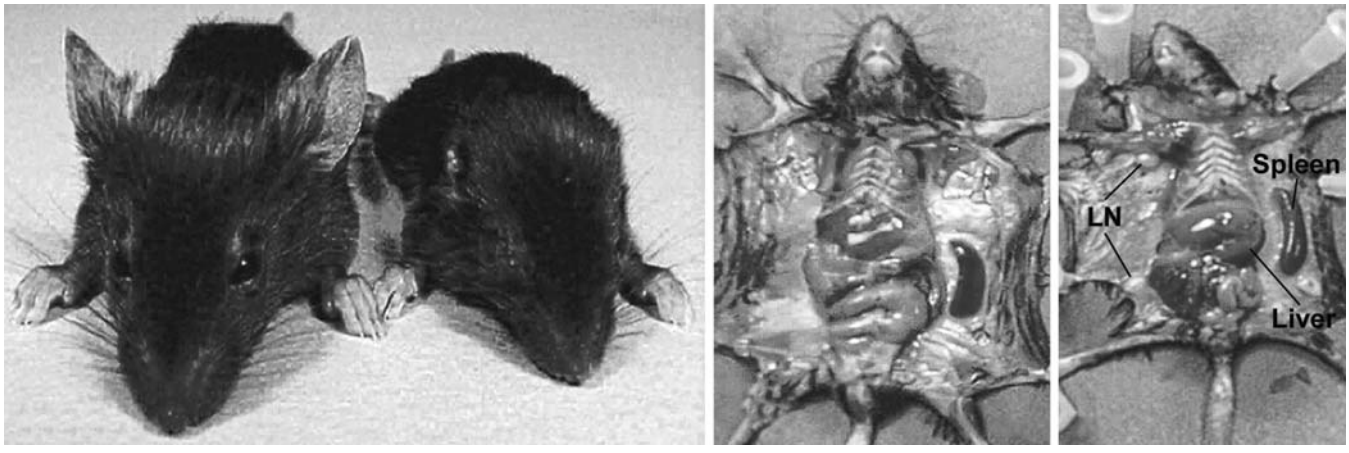

Figure 32.8 (Left panel) Wild-type (*left*) and scurfy (*right*) littermates, age 21 days. Note the small size of the scurfy mouse, the small and thickened ears, and the scaling of the feet. Compared to the wild-type mouse (middle panel), the scurfy mouse (right panel) has hepatosplenomegaly and enlarged lymph nodes (LN). Scurfy mice are deficient in FOXP3. (See Color Plate.)

IMMUNOLOGICAL FINDINGS

After a systematic study of these mice, Godfrey et al. suggested that "the scurfy disease may be the result of immune dysfunction rather than being a classic immune deficiency" (Godfrey et al., 1991b). This was supported by the observation that the thymus of young *sf* mice, although small, is densely populated with lymphocytes and has a distinct cortex and medulla. As the disease progresses, the thymic cortex is rapidly depleted of lymphocytes, and in mice older than 25 days, the thymus is a "shrunken stromal remnant lacking any corticomedullary distinction" (Godfrey et al., 1991b). The lymph nodes, spleen, and liver, however, are markedly enlarged from extramedullary hematopoiesis and cellular infiltrates. Transfer experiments demonstrated that the scurfy phenotype is mediated by T cells (Godfrey et al., 1994). T-cell subset depletion procedures strongly suggested that $CD4^+$ T cells are critical mediators of the lymphoproliferative disease in the scurfy mouse (Blair et al., 1994). Serum IgG and IgM are markedly elevated; IgA, which is absent in young normal mice, is demonstrable in male pups with the *sf* mutation (Godfrey et al., 1991a). Flow cytometric analysis of cell suspensions from the spleen or lymph nodes of *sf* mice reveal a characteristic increase in Mac-1^+ monocytic cells and a decrease in $B220^+$ cells; the latter population shows increased expression of B7.1 and B7.2 co-stimulatory molecules, suggesting a state of activation (Clark et al., 1999). The activity of *sf* T cells appears upregulated in vivo, as suggested by the spontaneous expression of CD69, CD25, CD80 (B7.1) and CD86 (B7.2). Although *sf* T cells are hyperresponsive to TCR ligation, they still require co-stimulation through CD28, albeit at decreased intensity (Clark et al., 1999). Following activation, $CD4^+$ T cells produce large amounts of granulocyte-macrophage colony-stimulating factor (>1,000-fold higher than wild-type $CD4^+$) as well as a number of other cytokines, including IL-2, IL-5, IL-6, IL-7, IL10, IFN-γ, and TNF-α (Clark et al., 1999; Kanangat et al., 1996). Interestingly, this hyperresponsiveness is resistant to suppression by inhibitors of tyrosine kinases, such as genistein and herbimycin A, and cyclosporin A (Clark et al., 1999). These observations suggest that the *sf* mutation interferes with the physiological downregulation of T-cell activation and may have implications for the immunosuppressive therapy of IPEX patients.

TRANSFER EXPERIMENTS IN *SF* MICE

The effect of transplantation of lymphoid organs and cell suspensions from *sf* (or wild-type) mice into athymic nude or severe combined immunodeficiency disease (SCID) mice (Blair et al., 1994; Godfrey et al., 1994) can be summarized as follows. First, transplanted *sf* thymus transfers the *sf* phenotype to nude and SCID mice. Second, euthymic (immunological competent) recipients of congenic *sf* thymus grafts remain clinically normal, as do all SCID and nude recipients of normal thymic transplants. Third, single-cell suspensions of thymus, lymph node, or spleen from *sf* mice transferred into histocompatible nude or SCID mice by intraperitoneal injection result in an *sf* phenotype in all recipients. Additional transfer experiments have demonstrated that the primary driver of the *sf* phenotype is the $CD4^+CD8^-$ T-cell population; $CD4^+$ but not $CD8^+$ lymph node T cells from *sf* mice transfer the disease to syngeneic host nude mice (Blair et al., 1994). Finally, if normal $CD4^+$ T cells are added to the $CD4^+$ *sf* T cells, the host nude mice do not develop the *sf* phenotype. These in vivo experiments suggest that *sf* is the result of dysregulated $CD4^+$ T cells, which express activation markers, excrete large quantities of cytokines, and are capable of inducing autoimmune disorders.

MUTATIONS AFFECTING THE *FOXP3* GENE ARE THE CAUSE OF *SF*

The *sf* locus was originally mapped to a 1.7 Cm interval between *DXWas70* and *Otc* in the proximal region of the mouse X chromosome (Kanangat et al., 1996; Lyon et al., 1990). Through combining high-resolution genetic and physical mapping with large-scale sequence analysis, the gene responsible for the *sf* mutation was identified and designated as Foxp3 (Brunkow et al., 2001). The protein encoded by *Foxp3* is a member of the forkhead winged-helix family of transcriptional regulators and is highly conserved in different mammalian species. The spontaneous mutation of *Foxp3* in *sf* mice is a 2bp insertion in exon 8, resulting in a frameshift that leads to a truncated protein lacking the carboxy-terminal forkhead domain (Brunkow et al., 2001).

FOXP3 TRANSGENIC MICE

To examine the in vivo consequences of Foxp3 overexpression, several different Foxp3-transgenic mouse lines have been established, each with differing levels of transgene expression (Brunkow et al., 2001; Khattri et al., 2001). Each of these lines, when bred onto an otherwise wild-type background, resulted in a reduction of lymph node size and cellularity. The extent of this reduction correlated with transgene copy number and expression, with higher Foxp3 levels resulting in a greater reduction in lymph node and splenic cellularity. Both $CD4^+$ and $CD8^+$ lymphocytes were reduced in number, while thymic cellularity was unaffected. A transgenic line with 16 copies of the transgene and an expression level of Foxp3 that was approximately threefold higher than in normal controls had a 50 percent reduction in the number of peripheral blood $CD4^+$ T cells and a 75 percent reduction in $CD8^+$ T cells. Histologically, peripheral lymphoid organs lacked follicular structure and were without margins between follicular and interfollicular regions. Purified $CD4^+$ T cells from this transgenic line displayed reduced proliferative responses when activated in vitro and lacked IL-2 production. Thus, Foxp3 is capable of regulating the ability of $CD4^+$ T cells to respond to TCR-mediated signals. As a direct consequence, T-helper function is severely impaired in Foxp3 transgenic mice, resulting in failure to respond to T-dependent antigens (Kasprowicz et al., 2003).

Finally, the *sf* mouse model was instrumental in defining the characteristics of the regulatory T-cell lineage (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; see "FOXP3

and $CD4^+CD25^+$ Regulatory T Cells," above) and thus played a major role in understanding the immunological basis of IPEX. Using Foxp3-negative gene-targeted mice and a GFP-Foxp3 fusion–protein–reporter knock-in allele, it could be shown that expression of Foxp3 was highly restricted to αβ $CD4^+$ T cells and, irrespective of CD25 expression, correlated with suppressor activity (Fontenot et al., 2005).

CTLA-4- AND TGF-β1-DEFICIENT MICE

Two mouse models, neither with a human counterpart, show many similarities to the scurfy mouse. Ctla-4-deficient mice develop a lymphoproliferative syndrome similar to that of scurfy mice; however, the lymphocytic infiltrates tend to be more widespread, with accumulation of activated T cells in the heart, lungs, lymph nodes, thymus, spleen, liver, bone marrow, and pancreas. Mice are well until 2 weeks of age, then become ill and die by 3 to 4 weeks of age, probably of myocardial infarction due to massive lymphocytic infiltrates in the heart muscle (Tivol et al., 1995; Waterhouse et al., 1995). Ctla-4 is a cell-surface molecule expressed by activated T cells. When engaged by its ligand B7.1 or B7.2, Ctla-4 has an inhibitory effect on T-cell activation responses, including proliferation, cytokine secretion, and cell-surface protein expression (Chambers and Allison, 1999). Although there is no identified human syndrome caused by mutations of CTLA-4, it has been suggested that specific polymorphisms may increase the risk of developing Graves disease or autoimmune diabetes (Cosentino et al., 2002; Yung et al., 2002).

Transforming growth factor β1 (TGF-β1) belongs to a family of related growth factors that play diverse roles in cellular processes, including wound healing, hematopoiesis, and immune regulation. Tgf-β1-deficient mice develop normally until 2 weeks of age, when they begin to waste, and die by 3 to 4 weeks of age. Deficient mice also develop lymphocytic infiltrates into major organs, particularly the heart, lungs, and salivary glands, and die of cardiopulmonary symptoms secondary to massive lymphocytic infiltrates (Christ et al., 1994; Kulkarni et al., 1995; Shull et al., 1992). Tgf-β1 knockout mice develop high autoantibody titers to several nuclear antigens, including single- and double-stranded DNA (Yaswen et al., 1996). Mice conditionally lacking the transforming growth factor beta type II receptor (Tgf-βRII), one of the two cell-surface receptors for the TGF-β family of growth factors, also have a phenotype of lymphocytic infiltration and autoimmunity, but with a less fulminant course (Leveen et al., 2002).

CONCLUSION AND FUTURE DIRECTIONS

FOXP3 is the key mediator of regulatory T-cell development and function. Naturally occurring mutations in *FOXP*3 interfere with this process, resulting in the generation of unregulated autoaggressive T-lymphocyte clones and autoreactive antibody-producing B cells that are directly responsible for IPEX in humans and scurfy in mice, both lethal diseases. T-cell–directed immune suppression and aggressive supportive care are essential for stabilizing acutely ill patients, but at present hematopoietic stem cell transplantation is the only cure for patients with IPEX. Over the coming years, it is anticipated that careful evaluation of patients with IPEX and exploitation of animal models such as scurfy will lead to insights that not only answer important questions related to the care and treatment of IPEX but address more basic questions, including how FOXP3 expression and function are regulated, which FOXP3-regulated genes mediate the suppressive activity of regulatory T cells, and most importantly how Tregs functionally suppress target cells. These studies will have implications not only for patients with IPEX but also for those suffering from IPEX-like phenotypes, autoimmune diseases, graft-versus-host disease, or cancer.

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33.

RECURRENT FEVER SYNDROMES

Lori Broderick, Daniel L. Kastner, and Hal M. Hoffman

INTRODUCTION

The periodic fever syndromes are a heterogeneous group of disorders characterized by recurrent episodes of fever and localized inflammation, most commonly affecting the serosal membranes, joints, and skin (Table 33.1). Molecular genetic studies have revealed that these syndromes represent dysregulation of innate immunity. Consequently, the term "autoinflammatory disorders" has been used to describe this unique group (Doherty et al., 2011; Galon et al., 2000; Goldbach-Mansky et al., 2009; Hull et al., 2003; Masters et al., 2009; McDermott et al., 1999). In contrast to autoimmune disorders (e.g., systemic lupus erythematosus), autoinflammatory disorders notably lack high-titer autoantibodies and antigen-specific T cells. The predominance of monocytes and neutrophils as effector cells, rather than lymphocytes, underscores the key role of the innate immune system in the natural history of these disorders.

Advances in the past several years have significantly increased our understanding of these disorders, with the identification of four genes underlying six clinically distinct recurrent fever syndromes. The precise mechanism by which mutations in each of these genes causes recurrent fever and inflammation is still under investigation. However, data from both in vitro and in vivo models suggest that the dysregulation of innate immunity leads to overproduction of interleukin (IL)-1β, and the inflammatory phenotype. There is an emerging body of data indicating that both pyrin, the protein implicated in familial Mediterranean fever, and cryopyrin, the protein mutated in familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and neonatal-onset multisystem inflammatory disorder, are regulators of IL-1β production, NF-κB activation, and leukocyte cell death, all critical components of innate immunity. Similarly, while mutations in the extracellular domain of the p55 TNF receptor, as observed in TNF receptor-associated periodic syndrome, lead to increased TNF signaling, there also appears to be dysregulated IL-1β production. Although mutations in mevalonate kinase, a key enzyme in cholesterol biosynthesis, were quite unexpected in hyperimmunoglobulinemia D with periodic fever syndrome, new data suggest a nexus between the mevalonate pathway and IL-1β regulation. These findings have significantly enhanced our understanding of the molecular pathways involved in innate immunity and led to substantial successes in the treatment of autoinflammatory disorders.

THE CRYOPYRINOPATHIES

The cryopyrinopathies––familial cold autoinflammatory syndrome (FCAS, MIM 120100), Muckle-Wells syndrome (MWS, MIM 191100), and neonatal-onset multisystem inflammatory disease (NOMID, MIM 607115, also called chronic infantile neurologic cutaneous and articular [CINCA] syndrome)––are a group of autosomal dominant autoinflammatory conditions consisting of episodic or fluctuating inflammation with associated cutaneous and synovial symptoms. A large family with what is now called FCAS was described first (Kile et al., 1940), and Muckle and Wells published a family with their eponymous syndrome in 1962 (Muckle et al., 1962). NOMID/CINCA was first recognized as a clinical entity in the early 1980s (Hassink et al., 1983; Prieur et al., 1981), although it was not initially apparent that this condition is a genetic disorder due to decreased reproductive fitness in patients carrying the diagnosis.

Between 1999 and 2000, genetic markers associated with FCAS, MWS, and a clinical overlap syndrome sharing features of both conditions were independently mapped to the distal

Table 33.1 CHARACTERISTICS OF THE PERIODIC FEVER SYNDROMES

	FCAS	MWS	NOMID/CINCA	FMF	TRAPS	HIDS	PFAPA
GENE	*CI*AS1	CIAS1	CIAS1	MEFV	TNFRSF1A	MVK	UNKNOWN
Inheritance	Dominant	Dominant	De novo, dominant	Recessive	Dominant	Recessive	Unknown
Ethnicity	Mostly European	Mostly European	Any ethnicity	Turkish, Armenian, Arab, Jewish, Italian, Greek, other	Any ethnicity	Dutch, French, other European	Any ethnicity
Duration of attacks	12–24 hours	2–3 days	Continuous	12–72 hours	>7 days	3–7 days	3–5 days, regular periodicity
Cutaneous	Urticaria-like rash induced by cold temperatures	Urticaria-like rash	Urticaria-like rash	Erysipeloid erythematous rash on lower leg, ankle, foot	Migratory rash, often associated with underlying myalgia	Maculopapular rash on trunk and limbs, urticaria	Not common
Abdominal	Nausea	Abdominal pain	Not common	Sterile peritonitis, constipation	Peritonitis, diarrhea or constipation	Severe pain, vomiting, diarrhea, rarely peritonitis	Abdominal pain, nausea
Pleural	Not seen	Rare	Rare	Common	Common	Rare	Not seen
Arthropathy	Polyarthralgia	Polyarthralgia, oligoarthritis	Epiphyseal overgrowth, contractures, intermittent or chronic arthritis	Monoarthritis, occasionally protracted arthritis in knee or hip	Arthritis in large joints, arthralgia	Arthralgia, symmetrical polyarthritis	Arthralgia
Ocular	Conjunctivitis	Conjunctivitis, episcleritis	Uveitis, conjunctivitis, progressive vision loss	Rare	Conjunctivitis, periorbital edema	Uncommon	Not seen
Neurological	Headache	Headache, sensorineural deafness	Sensorineural deafness, chronic aseptic meningitis, mental retardation, headache	Rare	Rare	Headache	Not seen
Lymph nodes/ spleen	Not seen	Rare	Hepatosplenomegaly, adenopathy	Splenomegaly more common than lymphadenopathy	Splenomegaly more common than adenopathy	Cervical adenopathy more common than splenomegaly	Cervical adenopathy
Vasculitis	Not seen	Not seen	Occasional	Henoch-Schönlein purpura, polyarteritis nodosa	Henoch-Schönlein purpura, lymphocytic vasculitis	Cutaneous vasculitis common, rarely Henoch-Schönlein purpura	Not seen
Amyloidosis	Rare	Occurs in ~25%	May develop in a portion of patients reaching adulthood	Risk depends on *MEFV* and *SAA* genotypes, therapy, other factors	Occurs in ~10%	Case reports	Not seen
Treatment[1]	Anakinra, canakinumab*, rilonacept*, NSAIDs	Anakinra, canakinumab*, rilonacept*, NSAIDs	Anakinra, canakinumab*	Colchicine*, anakinra, etanercept	Corticosteroids, anakinra, etanercept, NSAIDs	NSAIDs, etanercept, anakinra	Corticosteroids, tonsillectomy investigational

[1]Treatment of autoinflammatory disorders is evolving. Asterisks (*) denote approved therapies, although not in all countries. Other treatments, although they may be considered standard of care, have yet to be approved by regulatory agencies.

FCAS, familial cold autoinflammatory syndrome; FMF, familial Mediterranean fever; HIDS, hyperimmunoglobulinemia D with periodic fever syndrome; MWS, Muckle-Wells syndrome; NOMID, neonatal-onset multisystem inflammatory disorder; NSAIDs, nonsteroidal anti-inflammatory drugs; TRAPS, TNF receptor-associated periodic syndrome.

region of the long arm of chromosome 1 (Cuisset et al., 1999; Hoffman et al., 2000; McDermott et al., 2000), suggesting that all three disorders were linked to the same gene. In 2001, several missense mutations associated with FCAS and MWS were identified in an exon of a newly discovered gene denoted *CIAS1* (cold-induced auto-inflammatory syndrome-1) (Hoffman et al., 2001a). This gene, which was also called *PYPAF1* (Manji et al., 2002), *NALP3* (Aganna et al., 2002a), and *CATERPILLER1.1* (O'Connor et al., 2003), is now referred to as *NLRP3* (Ting et al., 2008). Similarities between clinical features of NOMID/CINCA and MWS led to the discovery, in 2002, of de novo missense mutations in *CIAS1* in many patients with NOMID/CINCA (Aksentijevich et al., 2002; Feldmann et al., 2002). Currently, over 85 mutations linked to cryopyrinopathies have been found in *NLRP3.* An updated list of mutations is available at the Infevers website (http://fmf.igh.cnrs.fr/infevers/) (Milhavet et al., 2008). Several mutations are shared by more than one condition, and many patients evince symptoms that fall between the established diagnostic boundaries, creating a phenotypic spectrum (Aksentijevich et al., 2007; Neven et al., 2004). Variability in phenotype among patients with the same mutation suggests that other genetic or environmental factors may influence disease severity.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF THE CRYOPYRINOPATHIES

Familial Cold Autoinflammatory Syndrome (FCAS)

FCAS, also called familial cold urticaria, familial polymorphous cold eruption, and cold hypersensitivity, is considered the mildest of the cryopyrinopathies. Symptoms include episodes of fever, urticaria-like rash, and arthralgia, and, less commonly, drowsiness, fatigue, headache, nausea, and extreme thirst (Hoffman et al., 2001b; Johnstone et al., 2003; Wanderer et al., 2004). Febrile attacks are often brought on by generalized exposure to cold temperatures. In many patients, less than an hour of mild cold exposure is needed to precipitate symptoms (Hoffman et al., 2001b). Approximately 2 hours later, patients experience symptoms that last on average about 12 hours. Longer and more extreme exposure to cold correlates with more severe symptoms. In some individuals, attacks occur nearly daily, often worse in the evening and resolving by morning. Most patients have their first symptoms by 6 months of age, and more than half develop the characteristic rash within the first few days of life.

The rash associated with FCAS (Plate 33.I) can present as petechiae, erythematous patches, or confluent plaques, usually beginning on the face or extremities and spreading (Johnstone et al., 2003). Although the rash appears hive-like, skin biopsies reveal infiltrates of predominantly neutrophils but also eosinophils and lymphocytes that are often perivascular or perieccrine with little evidence of mast cell degranulation, indicating that the rash is not typical urticaria, but a neutrophilic urticarial dermatosis (Hoffman et al., 2001b; Kolivras et al., 2011).

Polyarthralgia and swelling involving the hands, knees, and ankles, and occasionally the feet, wrists, and elbows, usually accompany flares. Occasionally, some patients develop a nonerosive arthropathy with deformities of the metacarpophalangeal and proximal interphalangeal joints (Commerford et al., 1977). Some patients also complain of ocular symptoms, including pain, redness, watering, and blurred vision (Hoffman et al., 2001b). While FCAS is the mildest of the phenotypes, the disease has considerable effect on quality of life (Stych et al., 2008) and is rarely associated with amyloidosis.

Muckle Wells Syndrome (MWS)

The first published description of MWS was a 1962 report on a Derbyshire kindred (Muckle et al., 1962). Affected individuals suffered from "aguey bouts" of inflammation consisting of fever, malaise, an urticaria-like rash, and stabbing pains in the large joints. In addition, many affected members of the family developed bilateral sensorineural hearing loss and renal amyloidosis. Attacks were not usually linked to cold temperatures and tended to be longer and more severe than those associated with FCAS.

Age of onset in MWS ranges from infancy to adulthood, but most patients experience their first attack by adolescence. MWS-associated episodes typically last from 24 to 48 hours, but many patients have daily symptoms with a similar circadian pattern of worsening in the evening (Cuisset et al., 1999; Muckle, 1979; Muckle et al., 1962). In addition to the symptoms noted above, patients may also have headache, abdominal pain, conjunctivitis, and episcleritis (Cuisset et al., 1999; Watts et al., 1994). Skin manifestations similar to those associated with FCAS usually accompany episodes (Plate 33.I). Although arthralgia is much more common than arthritis, oligoarticular synovitis and sterile pyogenic arthritis occur occasionally (Schwarz et al., 1989; Watts et al., 1994). Approximately three fourths of patients develop sensorineural hearing loss, usually beginning in childhood with high-frequency hearing loss (Schwarz et al., 1989). Mucosal thickening and maxillary sinus hypoplasia is also commonly observed on magnetic resonance imaging (MRI; Ahmadi et al., 2011).

Systemic amyloidosis develops in approximately one third of adult patients due to the deposition of a fragment of serum amyloid A (SAA) in target organs, namely the kidneys. SAA is an acute phase reactant produced by the liver and found at high levels in the serum during inflammatory attacks. Patients with amyloid deposition in the kidneys progress from albuminuria to the nephrotic syndrome and ultimately to renal failure. Other target organs include the thyroid, adrenals, spleen, and testes (Schwarz et al., 1989). Hearing loss does not appear to be due to amyloid deposition in the inner ear or auditory nerve, and the current hypotheses focus on inflammation of the inner ear.

Neonatal onset multisystem inflammatory disease/ chronic infantile neurologic cutaneous and articular syndrome (NOMID/CINCA)

NOMID/CINCA is the most severe of the cryopyrinopathies. In addition to the rash, fever, arthralgia, headache, hearing loss, and amyloidosis seen in MWS, NOMID/CINCA

patients may have a distinct, deforming arthropathy (Hashkes et al., 1997; Hassink et al., 1983; Prieur, 2001; Prieur et al., 1981), as well as chronic aseptic meningitis, intellectual impairment, and significant eye involvement. Approximately 20 percent of patients die before age 20 years (Hashkes et al., 1997; Prieur et al., 1987).

The rash associated with NOMID/CINCA is similar to that associated with FCAS and MWS and is nearly always present to some degree (Prieur, 2001). Articular symptoms are variable (Hashkes et al., 1997; Prieur, 2001; Prieur et al., 1987), with some patients exhibiting mild swelling and pain but no radiographic changes, while others have severe deformities involving symmetrical overgrowth of the epiphyses and growth cartilage of the long bones, especially prominent in the knees, ankles, elbows, and hands. The latter changes may occur within the first year of life and contractures may severely restrict movement (Hashkes et al., 1997; Prieur, 2001; Prieur et al., 1987). Joint radiographs from severe cases may show enlarged, irregular ossification of the epiphyses of the femur and tibia and enlargement of the patella, often with a "breadcrumb" appearance. There may be early growth-plate closure and shortening of the long bones, suggesting abnormal endochondral bone growth (Hashkes et al., 1997; Kaufman et al., 1986). Skull radiographs may show frontal bosselation, delayed closure of the anterior fontanelle, and increased cranial volume. Synovial biopsy usually shows only modest inflammation.

The neurosensory manifestations of NOMID/CINCA are potentially of great concern. Many patients develop chronic aseptic meningitis, with increased cerebrospinal fluid (CSF) pressure, an elevation in CSF protein concentration, and a pleocytosis consisting mostly of polymorphonuclear leukocytes (Torbiak et al., 1989). Brain imaging studies may reveal ventricular dilatation, cerebral atrophy, and prominent sulci (Prieur, 2001). Although some patients perform well at school, other children with NOMID/CINCA have developmental delays and learning deficits and still others exhibit mental retardation (Dollfus et al., 2000; Torbiak et al., 1989). As in MWS, sensorineural hearing loss is frequent and is associated with cochlear enhancement on MRI (Ahmadi et al., 2011). Ocular involvement may range from conjunctivitis to anterior or posterior uveitis, sometimes leading to blindness (Dollfus et al., 2000). Episcleritis and corneal infiltrates have also been observed. Funduscopic examination frequently reveals optic disc edema, papilledema, or optic atrophy (Terrada et al., 2011).

Other findings include hepatosplenomegaly and lymphadenopathy, as well as vasculitis, thrombosis, and the aforementioned amyloidosis (Hashkes et al., 1997; Prieur, 2001; Prieur et al., 1987; Torbiak et al., 1989). Profound growth delay and reduced reproductive potential are also common.

LABORATORY FINDINGS IN THE CRYOPYRINOPATHIES

Laboratory features of the cryopyrinopathies include an accelerated erythrocyte sedimentation rate (ESR), thrombocytosis, and anemia of chronic disease at baseline and polymorphonuclear leukocytosis that increases during episodes (Hoffman et al., 2004). Elevated acute phase reactants, such as the C-reactive protein (CRP) and SAA, are also observed chronically without therapy. Elevated S100A12 levels have also been described (Kuemmerle-Deschner et al., 2011; Wittkowski et al., 2008). High-titer autoantibodies are not observed, although some patients do have modest titers of anti-cardiolipin antibodies.

MOLECULAR BASIS OF THE CRYOPYRINOPATHIES: THE NLRP3 GENE

NLRP3 consists of nine exons, encoding a 3105 bp potential open reading frame based on a second start site. An open reading frame of 3111 bp based on the first start site 6 bp upstream has resulted in some confusion when naming specific mutations due to the two additional amino acids. There is extensive alternative splicing of exons 4–9, resulting in multiple alternative splice forms of unclear significance (Hoffman et al., 2001a). Expression analyses detect the cryopyrin message primarily in leukocytes and chondrocytes (Feldmann et al., 2002; Hoffman et al., 2001a), although recent data suggest that is it is expressed at lower levels in several tissue and cell types with potential functional significance (Kummer et al., 2007; McCall et al., 2008; Shigeoka et al., 2010).

The protein product of *NLRP3* was named cryopyrin to emphasize the existence of an N-terminal PYRIN domain, initially described in the pyrin protein (see the section on familial Mediterranean fever), as well as the association with cold-induced symptoms in FCAS (Hoffman et al., 2001a). The PYRIN domain (Bertin et al., 2000), also known as PYD (Martinon et al., 2001), PAAD (Pawlowski et al., 2001), or DAPIN (Staub et al., 2001), has now been recognized in a total of over 20 human proteins involved in the regulation of inflammation and apoptosis (Harton et al., 2002; Tschopp et al., 2003). Computational modeling and subsequent nuclear magnetic resonance (NMR) spectroscopy have demonstrated that the PYRIN domain is the fourth member of the death domain-fold superfamily (Eliezer, 2003; Fairbrother et al., 2001; Hiller et al., 2003; Liepinsh et al., 2003; Liu et al., 2003; Richards et al., 2001), which also includes death domains, death effector domains, and caspase-recruitment domains (CARDs). All four assume a six alpha-helix three-dimensional structure that facilitates homotypic interactions through electrostatic charge interactions. Thus, the PYRIN domain of cryopyrin is a docking motif that facilitates cognate interactions with other PYRIN domain proteins.

Besides the PYRIN domain (amino acids [a.a.] 13–83), cryopyrin contains a central NACHT domain (a.a. 217–533) (Koonin et al., 2000), named for its presence in neuronal apoptosis inhibitor protein (*NA*IP), the major histocompatibility complex (MHC) class II transactivator (*C*IITA), the incompatibility locus protein from *Podospora anserina* (*H*ET-E), and mammalian telomerase-associated proteins (*T*P1). At the C-terminus of cryopyrin are seven leucine-rich repeats (LRRs) (a.a. 697–920). This arrangement of PYRIN-NACHT-LRRs is found in at least 14 proteins referred to as NLRPs, or NALPs in the human genome (Tschopp et al., 2003), and the

C-terminal domains, NACHT-LRR, are found in many other proteins in the NLR protein family (Ting et al., 2008).

FUNCTION OF CRYOPYRIN, THE NLRP3 GENE PRODUCT

The PYRIN domain of cryopyrin has been shown to interact specifically with ASC (apoptosis-associated speck-like protein with a CARD), a bipartite adaptor consisting solely of an N-terminal PYRIN domain and a C-terminal CARD in tandem (Dowds et al., 2003; Gumucio et al., 2002; Manji et al., 2002). Through its CARD, ASC binds caspase 1 (also known as IL-1β converting enzyme [ICE]) and other adaptor proteins. This macromolecular complex of cryopyrin, ASC, and caspase 1, and possibly a fourth protein called Cardinal, has been denoted the inflammasome (Agostini et al., 2004; Martinon et al., 2004b). Activation of the inflammasome leads to the cleavage of caspase 1 into enzymatically active p20 and p10 subunits (Agostini et al., 2004; Chae et al., 2003; Martinon et al., 2002, 2004b; Srinivasula et al., 2002; Stehlik et al., 2003; Wang et al., 2002). Activated caspase 1, in turn, cleaves IL-1β from its 31 kDa precursor form to its 17 kDa biologically active fragment, which is a potent mediator of fever and inflammation (Fig. 33.1). Studies in various transfection systems indicate that cryopyrin potentiates inflammation through its interaction with ASC. Cryopyrin has been shown to regulate IL-1β secretion (Stehlik et al., 2003; Wang et al., 2002), NF-κB activation (Dowds et al., 2003; Gumucio et al., 2002; Manji et al., 2002; O'Connor et al., 2003; Stehlik et al., 2002), and cell death (Dowds et al., 2003; Duncan et al., 2009; Fujisawa et al., 2007; Willingham et al., 2009; Willingham et al., 2007).

The NACHT domain contains seven conserved motifs, including an ATPase-specific P-loop and a Mg^{++} binding site, and is involved in protein oligomerization (Koonin et al., 2000). This domain has been found to have ATPase activity that is important to its function (Duncan et al., 2007). Disease-associated mutations are found almost exclusively in the NACHT domain, suggesting an important role for this motif in the function of cryopyrin.

LRRs are common components of the extracellular domains of Toll-like receptors (TLRs), as well as several cytoplasmic proteins known as NLRs. The LRRs of TLRs appear to recognize pathogen-associated molecular patterns (PAMPs), common molecules in bacterial cell walls, such as peptidoglycan and lipopolysaccharide (Chamaillard et al., 2003; Girardin et al., 2003; Inohara et al., 2002). Upon contact with a PAMP, TLRs transduce signals into the cell to upregulate proinflammatory mediators such as co-stimulatory molecules, cytokines, and inducible nitric oxide synthase (iNOS). LRRs on cytoplasmic proteins such as cryopyrin may serve a similar purpose in detecting intracellular bacteria or bacterial products. Several PAMPs have been shown to activate formation of the cryopyrin inflammasome (Lamkanfi et al., 2009; Martinon et al., 2004a). However, most of the activators of the cryopyrin inflammasome, such as various microorganisms, particles, and danger signals, are not thought to interact directly with cryopyrin. Instead, mechanisms involving potassium efflux, generation of reactive oxygen species, and membrane disruption have been proposed to mediate inflammasome activation (Hoffman et al., 2011).

Under normal conditions, cryopyrin is kept from activating caspase 1 in at least two possible ways. First, pyrin may compete with cryopyrin for binding to ASC (Dowds et al., 2003),

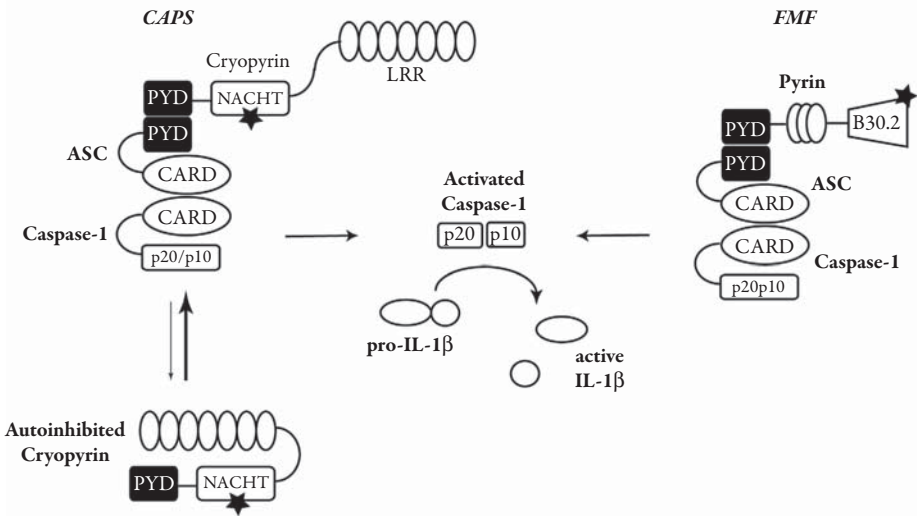

Figure 33.1 Enhanced production of IL-1β in cryopyrin-associated periodic disorders (CAPS) and familial Mediterranean fever (FMF). (*Left*) Formation of the cryopyrin (NLRP3) inflammasome. At baseline, interaction of the leucine-rich repeats (LRR) with the NACHT domain of cryopyrin prevents assembly of the complex. Mutations in the NACHT domain associated with familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID) may alter the LRR–NACHT interaction in such a way that the threshold of activation is diminished. Assembly of the inflammasome leads to the interaction of the catalytic domains (p20/p10) of caspase 1 molecules, leading to autocatalysis, release of p20 and p10, and subsequent IL-1β activation. (*Right*) PYRIN domain of pyrin interacts with ASC and caspase 1, leading to the cleavage of caspase 1 into its enzymatically active subunits, again resulting in IL-1β cleavage and activation. Stars denote the presence of mutations.

preventing formation of the cryopyrin inflammasome. Second, studies of LRR deletion mutants of both cryopyrin and NLRP1 (Agostini et al., 2004; Martinon et al., 2002), a related protein, suggest that the LRRs have an autoinhibitory function, maintaining the protein in an inactive state, although there are conflicting data in mice (Hoffman et al., 2010). Upon activation, the LRRs may release the rest of the protein from inhibition, allowing formation of the cryopyrin inflammasome.

MUTATION ANALYSIS IN THE CRYOPYRINOPATHIES

Nearly all of the known mutations occur in exon 3, encoding the NACHT domain. Although the crystal structure of cryopyrin has not been solved, computational modeling suggests that many of the disease-causing mutations are located along the nucleotide binding cleft or near a region that senses when a nucleotide is bound (Neven et al., 2004) or on a predicted surface of the protein domain, suggesting an effect on protein–protein interactions (Aksentijevich et al., 2007).

Mutation-associated changes in cryopyrin appear to increase its proinflammatory properties, perhaps by increasing the binding affinity of cryopyrin for ASC or rendering the protein more labile, so that minor stimuli (such as cold temperatures and low-dose lipopolysaccharide [LPS]) trigger inflammasome activation and IL-1β release, as observed in FCAS monocytes (Rosengren et al., 2007; Stack et al., 2005). In addition, there is no requirement for ATP as a second stimulus of IL-1β release from cultured monocytes of all mutation-positive cryopyrinopathy patients (Brydges et al., 2009; Gattorno et al., 2007). Disease-associated mutations have also been associated with NF-κB activation (Dowds et al., 2003; Manji et al., 2002), increased cathepsin B-mediated cell death known as pyronecrosis (Fujisawa et al., 2007; Saito et al., 2008; Willingham et al., 2007), an altered redox state that is proinflammatory (Tassi et al., 2010), and increased protein kinase activity postulated to be involved in the bone phenotype (Almeida et al., 2011). Disease-associated mutations may be considered gain-of-function genetic changes, consistent with the dominant inheritance patterns observed in the cryopyrinopathies.

There is some degree of genotype–phenotype correlation with specific mutations commonly associated with mild or severe disease, but there can be extensive phenotypic overlap with specific mutations or with variants within close proximity based on predicted primary, secondary, or tertiary structure (Aksentijevich et al., 2007). Almost all of the disease-associated *NLRP3* variants described are missense mutations. Many patients with a classic phenotype do not have easily identifiable mutations, suggesting the involvement of additional genes, and there is now considerable evidence for somatic mosaicism of *NLRP3* mutants in many patients previously thought to be mutation negative (Saito et al., 2008; Tanaka et al., 2011).

STRATEGIES FOR THE DIAGNOSIS OF THE CRYOPYRINOPATHIES

The diagnosis of these disorders is often a combination of clinical observations and genetic testing. Common features suggestive of the cryopyrinopathies include fever, urticaria-like rash, arthralgia, headache, childhood onset, and chronically increased CRP. For FCAS, a clinical diagnosis may be established by the presence of fever and rash induced by generalized cold exposure and at least four of the following: short (<24 hour) episodes, autosomal dominant inheritance, age of onset less than 6 months, conjunctivitis during attacks, and absence of deafness, periorbital edema, lymphadenopathy, and serositis. The absence of a positive ice cube test differentiates FCAS from typical cold urticaria (Johnstone et al., 2003). Longer episodes, hearing loss, lack of association with cold temperatures, and amyloidosis are more suggestive of MWS (Muckle, 1979), whereas aseptic meningitis, uveitis, and the characteristic arthropathy support the diagnosis of NOMID/CINCA (Hashkes et al., 1997; Neven et al., 2004; Prieur, 2001).

Disease severity may vary extensively, even among individuals harboring the same mutations, indicating that other factors influence phenotype. Some patients blur the boundaries between diagnoses, with some features of FCAS, such as cold sensitivity, and other findings consistent with MWS, such as amyloid nephropathy (Aganna et al., 2002a; Dode et al., 2002c; Hoffman et al., 2001a; Neven et al., 2004). Other patients may represent an overlap between MWS and NOMID/CINCA at the more severe end of the phenotypic continuum (Aksentijevich et al., 2002, 2007; Dode et al., 2002c; Feldmann et al., 2002; Granel et al., 2003; Lieberman et al., 1998; Neven et al., 2004; Rosen-Wolff et al., 2003; Saito et al., 2008; Tanaka et al., 2011).

Genetic testing is not necessary but can provide an unequivocal diagnosis, if positive. However, the sensitivity is not 100 percent, and only about half of patients meeting clinical criteria for NOMID/CINCA have demonstrable mutations in *NLRP3* (Aksentijevich et al., 2002; Neven et al., 2004). While some of these patients have been found to have somatic mutations, it is likely that other mutation-negative patients have defects in related genes. There are some mutations with reduced penetrance or atypical phenotypes, including V198M and R488K, and some variants with unknown significance such as Q703K (Aksentijevich et al., 2007; Arostegui et al., 2004; Ting et al., 2007; Verma et al., 2008).

Recently, patients with some phenotypic overlap with the cryopyrinopathies (cold-induced fever and arthralgia, but not urticaria-like rash) have been found to have mutations in the structurally related gene *NLRP12*. However, the pathogenic mechanisms appear to be different from that of NLRP3-associated diseases (Borghini et al., 2011; Jeru et al., 2008, 2011).

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

NLRP3 mutations are inherited in an autosomal dominant fashion, a pattern recognized early in families with FCAS and MWS (Johnstone et al., 2003; Kile et al., 1940; Muckle et al., 1962). Most NOMID/CINCA cases are sporadic, resulting from de novo mutations in the children of otherwise healthy individuals, although a small number of familial cases have been documented (Feldmann et al., 2002).

Thus far, most mutations in *CIAS1* show a high degree of penetrance, such that carriers nearly always show some clinical manifestations, although there are exceptions as described above. Again, it should be noted that family members with the same mutations may not exhibit all the same signs and symptoms with equal severity. Prenatal diagnosis of the cryopyrinopathies is certainly feasible but requires careful genetic counseling and consideration of the effectiveness of recent therapies.

TREATMENT AND PROGNOSIS

Patients with the cryopyrinopathies generally do not respond well to colchicine (Hoffman et al., 2001b). Corticosteroids are helpful in some cases (Tanaka et al., 2011), but they carry a high risk of side effects and often have minimal effect on the arthropathy or central nervous system manifestations of NOMID/CINCA (Hashkes et al., 1997; Prieur, 2001). NSAIDs offer some relief for arthralgia and constitutional symptoms but do little to control overall inflammation. TNF-α and IL-6 inhibitors have also been used in cryopyrinopathy patients with an incomplete response (Matsubara et al., 2006).

The most widely used and successful therapies for the cryopyrinopathies are IL-1–targeted therapies. Initial reports with IL-1 receptor antagonist in FCAS and MWS patients demonstrated essentially complete remission of all disease symptoms and normalization of systemic inflammatory markers (Hawkins et al., 2003, 2004; Hoffman et al., 2004). Larger open-label studies with daily anakinra (100 mg SQ) in cryopyrinopathy patients (Kuemmerle-Deschner et al., 2011; Leslie et al., 2006; Ross et al., 2008) have replicated the initial case reports, and longer studies have demonstrated sustained response and significantly improved quality of life (Lepore et al., 2010), sometimes even with lower or every-other-day dosing. However, the most impressive and life-altering response to anakinra has been observed in patients with NOMID/CINCA. Uveitis, rash, headache, and fever resolved, and pathologically elevated cerebrospinal pressure dropped significantly. Growth, development, hearing, and leptomeningeal lesions also improved in many patients with anakinra (1–2 mg/kg/day; Goldbach-Mansky et al., 2006). Other long-term studies have demonstrated similar impressive and sustained responses, although higher doses (up to 10 mg/kg/day) may be required in infants (Neven et al., 2010). It will be important to continue to follow children with NOMID/CINCA on anakinra to observe long-term academic performance and physical growth, as well as to determine whether very early treatment can avert joint deformities.

Two additional IL-1–targeted therapies have been applied to cryopyrinopathy patients with similar efficacy. Rilonacept, a fusion protein also known as IL-1 TRAP, demonstrated remarkable efficacy with weekly subcutaneous dosing in a placebo-controlled trial with more than 40 adult and adolescent FCAS and MWS patients (Goldbach-Mansky et al., 2008; Hoffman et al., 2008), while canakinumab, a monoclonal antibody to IL-1β, was effective when given every 2 months in more than 40 adults and children (Kuemmerle-Deschner et al., 2011; Lachmann et al., 2009). Both are now approved for use in cryopyrin-associated periodic syndromes.

Untreated, FCAS appears not to be associated with long-term sequelae, save for a few reports of renal amyloidosis occurring late in life in a few families (Hoffman et al., 2001b). Prognosis in MWS patients is highly dependent on the development of amyloidosis, which occurs in approximately one third of individuals. Many MWS patients develop significant bilateral sensorineural deafness. NOMID/CINCA patients often have a reduced life expectancy. Currently, about 20 percent die before the age of 20 of infection or amyloidosis (Hashkes et al., 1997; Prieur et al., 1987). Morbidities include intellectual impairment, vision loss, sensorineural hearing loss, and physical disability due to contractures.

Early and continued treatment with IL-1–targeted therapy decreases the risk of developing amyloidosis in cryopyrinopathy patients, possibly averting or delaying renal failure and the attendant complications. The role of IL-1 inhibition in reversing preexisting deficits depends on the circumstances. Aggressive treatment soon after the discovery of proteinuria has been shown to reverse the progression to renal failure (Thornton et al., 2007), and some patients have had improvement in their hearing after starting therapy (Rynne et al., 2006). Primary side effects from these drugs are injection-site reactions and increased risk of standard viral and bacterial infections, particularly *Streptococcus*.

ANIMAL MODELS

Studies using NRLP3 knockout mice established a role for the cryopyrin inflammasome in many disease models. However, the cryopyrinopathies are autosomal dominant gain-of-function mutations. Knock-in mice were developed that have proven to be fairly accurate models of human disease, with neutrophilic inflammation in the skin and other tissues and similar ex vivo responses (Brydges et al., 2009; Meng et al., 2009). Brydges et al. showed that murine disease is inflammasome dependent, partially IL-1 dependent, and independent of adaptive immunity. Meng et al. suggested a role for T_H17 cells and IL-17 in the cutaneous pathology, which is supported by recent human data (Lasiglie et al., 2011). Additional studies with these mice are under way to delineate further pathogenic mechanisms.

FAMILIAL MEDITERRANEAN FEVER

Although molecular genetic data suggest that founder mutations causing familial Mediterranean fever (FMF, MIM 249100) arose many centuries ago (International FMF Consortium, 1997), the first recognizable report of a case of FMF was published in 1908 (Janeway et al., 1908), and the first series of 10 cases of "benign paroxysmal peritonitis" was published in 1945 (Siegal, 1945). The predominance of case descriptions in North African and Iraqi Jewish populations led to the widely accepted "familial Mediterranean fever" nomenclature (Heller et al., 1958; Sohar et al., 1967), although it should be noted that other names, such as recurrent polyserositis,

recurrent hereditary polyserositis, and periodic disease, are sometimes used.

The high carrier frequencies in these populations gave rise to pseudodominance; however, careful segregation analysis indicated that FMF is recessively inherited, with incomplete penetrance (Sohar et al., 1962, 1967). Subsequent studies described in detail the serosal, synovial, and cutaneous manifestations of FMF and elucidated the connection between FMF and systemic AA amyloidosis (Sohar et al., 1967). Further epidemiological and family-based studies among non-Ashkenazi Jews and Armenians demonstrated extremely high frequencies of FMF (Rogers et al., 1989; Yuval et al., 1995), suggesting a possible heterozygote advantage, and identified ethnic differences in the susceptibility to amyloidosis (Meyerhoff, 1980; Pras et al., 1982).

Mapping of the FMF locus, *MEFV,* in 1992 (Pras et al., 1992) identification of the disease gene in 1997 (French FMF Consortium, 1997; International FMF Consortium, 1997) and the availability of genetic testing has broadened the clinical and ethnic/geographical spectrum of FMF, while studies of the *MEFV* gene product, pyrin, have shed new light on the regulation of cytokine production, NF-κB activation, and apoptosis, and have defined a functional domain present in over 20 human proteins involved in the regulation of inflammation.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF FMF

FMF is characterized by relatively discrete, usually 1- to 3-day episodes of fever with serositis, synovitis, or skin rash. In some patients attacks begin in infancy or very early childhood, and 80 to 90 percent of patients experience their first episode by age 20. Young children, especially less than 2 years of age, may present with fever alone (Padeh et al., 2010). The frequency of FMF attacks is highly variable, both among patients and for any given patient, with the interval between attacks ranging from days to years. Moreover, the type of attack (abdominal, pleural, arthritic) may vary over time. Some patients relate attacks to physical or emotional stress, although in many cases there is no obvious provocative event. There is a slight predominance of males in most series, possibly the result of underreporting in women or underrecognition because of confounding gynecological diagnoses. In some women, attacks may occur at a specific point in the menstrual cycle (Ben-Chetrit et al., 2001b) and sometimes remit during pregnancy (Schwabe et al., 1974; Sohar et al., 1967).

Attacks comprising fever and abdominal pain occur at some time in nearly all FMF patients and range from a dull aching pain to full-blown peritonitis, with boardlike rigidity, absent bowel sounds, and rebound tenderness. Constipation is common during the attacks, sometimes with a diarrhea occurring at the very end of the episode. Plain films may demonstrate air–fluid levels, and computed tomography (CT) may show thickened mesenteric folds, lymphadenopathy, splenomegaly, or a small amount of ascites (Zissin et al., 2003). On laparoscopy there may be a neutrophil-rich exudate. Repeated episodes may lead to peritoneal adhesions.

Pleurisy may occur alone with fever, or concurrently with abdominal pain. Pleuritic episodes are usually unilateral, with sharp, stabbing chest pain and, in some cases, diaphragmatic pain referred to the ipsilateral shoulder. Radiographic findings may include atelectasis (Brauman et al., 1987) due to splinting and, in a minority of cases, pleural effusion. Thoracentesis, when performed, yields a neutrophil-laden exudate. Pleural thickening sometimes develops after multiple attacks (Livneh et al., 1999).

Other forms of serosal inflammation may also be seen in FMF. Nonuremic pericarditis is much less common than peritoneal or pleural involvement (Kees et al., 1997; Turkish FMF Study Group, 2005; Tutar et al., 2003). Although small subclinical effusions are more frequent than symptomatic pericarditis, there have been rare reports of tamponade (Zimand et al., 1994). Unilateral acute scrotum occurs in about 5 percent of prepubertal boys with FMF (Livneh et al., 1994a; Majeed et al., 2000b), resulting from inflammation of the tunica vaginalis, an embryological remnant of the peritoneal membrane.

Joint involvement in FMF is particularly common among North African Jews (Brik et al., 2001; Pras et al., 1998) and has been related to the M694V homozygous genotype (Brik et al., 1999; Cazeneuve et al., 1999; Gershoni-Baruch et al., 2002), which is very frequent in this population. Acute monoarticular arthritis is most characteristic in FMF (Heller et al., 1966), often affecting the knee, ankle, or hip, and attacks tend to last longer than serosal episodes (often about 1 week), sometimes with large effusions, extreme pain, and inability to bear weight. Synovial fluid contains as many as 100,000 polymorphonuclear leukocytes/mm^3, but cultures are sterile. Soft tissue swelling may be apparent on X-rays taken during attacks, but erosive changes do not develop. A number of other less common oligo- or polyarticular patterns of arthritis may occur, especially in children (Ince et al., 2002; Majeed et al., 1997). Arthralgia is also very common. In the pre-colchicine era, about 5 percent of patients with acute monoarticular arthritis went on to develop protracted arthritis, usually affecting the hip (Sneh et al., 1977). In such cases, symptoms could last for several months, sometimes leading to secondary osteoarthritic radiographic changes and/or osteonecrosis, and requiring total hip replacement surgery. Chronic sacroiliitis may also occur in FMF, regardless of the HLA-B27 status or colchicine therapy (Brodey et al., 1975; Langevitz et al., 1997; Lehman et al., 1978).

Children with FMF frequently develop myalgia of the legs related to vigorous exertion (Majeed et al., 2000a). Much less commonly, FMF patients may experience attacks of febrile myalgia, with excruciating muscle pain unrelated to exertion, that can last from a few days to several weeks (Langevitz et al., 1994; Sidi et al., 2000). During these episodes the creatine kinase is normal, the ESR is prolonged, and the electromyogram shows nonspecific myopathic changes. Histologic data suggest that febrile myalgia is a form of vasculitis. Other forms of vasculitis, including Henoch-Schönlein purpura and polyarteritis nodosum, are also seen at increased frequency in FMF (Gedalia et al., 1992; Ozdogan et al., 1997; Ozen, 1999; Ozen et al., 2001; Rawashdeh et al., 1996; Tekin et al., 1999; Tinaztepe et al., 1997).

The most characteristic cutaneous lesion of FMF is erysipeloid erythema (Plate 33.II), a sharply demarcated, erythematous, warm, tender, swollen area 10 to 15 cm in diameter occurring unilaterally or bilaterally, usually on the dorsum of the foot, ankle, or lower leg (Azizi et al., 1976; Barzilai et al., 2000). On skin biopsy, there is a mixed perivascular infiltrate of polymorphonuclear leukocytes, histiocytes, and lymphocytes. As is the case for arthritis, the frequency of erysipeloid erythema may be increased among M694V homozygotes (Kone Paut et al., 2000).

Long-term complications of FMF necessitating preventive measures and prompt intervention include renal disease, which presents as glomerulonephritis or as a secondary manifestation of systemic amyloidosis (discussed below). FMF has been associated with crescentic rapidly progressive glomerulonephritis, mesangial IgA nephropathy, IgM nephropathy, and diffuse proliferative and exudative glomerulonephritis, with non-amyloid renal disease affecting up to 22 percent of patients (Akpolat et al., 2004; Cagdas et al., 2005). In these cases, glomerular injury is proposed to be secondary to chronic increased inflammation and may be diagnosed by persistent hematuria or proteinuria, with confirmation by renal biopsy.

As in the cryopyrinopathies, FMF attacks demonstrate elevated serum SAA levels, which lead to systemic amyloidosis in a subset of FMF patients. Deposition in the kidneys, adrenals, intestine, spleen, lung, and testes (Sohar et al., 1967) leads to organ dysfunction and failure. Patients with amyloid deposition in the kidneys may progress from albuminuria to complete renal failure, usually over the course of 3 to 5 years. Amyloid deposits in other organs may lead to malabsorption (Mor et al., 2003) and/or azoospermia and infertility (Ben-Chetrit et al., 1998a). Cardiac involvement, neuropathy, and arthropathy are very uncommon with the amyloidosis of FMF. The diagnosis is usually established by renal or rectal biopsy.

The amyloidosis of FMF usually occurs after the onset of inflammatory attacks (phenotype I) but rarely can occur as the first manifestation of FMF (phenotype II) (Sohar et al., 1967; Turkish FMF Study Group, 2005), perhaps due to subclinical elevations in the SAA. The overall risk of amyloidosis in FMF is the product of a complex interaction of factors, including the M694V/M694V genotype, which in most studies is associated with an increased risk of amyloidosis (Brik et al., 1999; Cazeneuve et al., 1999; Gershoni-Baruch et al., 2002, 2003; Majeed et al., 2002; Mansour et al., 2001). Other risk factors for amyloidosis include male gender, the SAA1 α/α genotype, a positive family history for amyloidosis, and colchicine noncompliance (Akar et al., 2003; Cazeneuve et al., 2000; Gershoni-Baruch et al., 2003; Turkish FMF Study Group, 2005). There are also geographical and secular effects on amyloid susceptibility (Akpolat et al., 2004; Cagdas et al., 2005; Touitou et al., 2007b), perhaps reflecting improvements in general medical care that may modify the SAA load from intercurrent illness.

Despite the underlying systemic inflammation, certain complications of chronic endothelial damage are rarely seen in FMF, including increased thrombotic events, and atherosclerosis, at least among patients receiving colchicine (Bilginer et al., 2008; Sari et al., 2007). Doppler imaging studies, however, have suggested that FMF patients have impaired left ventricular diastolic function and impaired coronary microvasculature (Caliskan et al., 2007; Grimaldi et al., 2006; Tavil et al., 2008), Whether these differences reflect the antiatherogenic effects of colchicine therapy (Yuksel et al., 2010), the populations studied, which trend toward younger patients (Ozcakar et al., 2011), or genotype–phenotype differences remains to be determined.

LABORATORY FINDINGS IN FMF

During the acute attacks of FMF, there is a leukocytosis, often with a left shift, an accelerated ESR, and increases in several serum acute phase proteins, including SAA, CRP, fibrinogen, haptoglobin, and the C3 and C4 complement components. The neutrophil-derived biomarker S100A12 is also significantly elevated (Kallinich et al., 2010). Urinalysis may demonstrate albuminuria and microscopic hematuria during attacks.

During the intercritical period, the white count and acute phase reactants may normalize, but in some patients there is biochemical evidence of persistent subclinical inflammation, including abnormalities of the ESR, SAA, CRP, S100A12, and fibrinogen (Duzova et al., 2003; Kallinich et al., 2010; Korkmaz et al., 2002; Tunca et al., 1999). Markers of hypercoagulability and fibrinolysis, including D-dimer, tissue plasminogen activator, and plasminogen activator inhibitor 1, may also be present (Demirel et al., 2008; Tayer-Shifman et al., 2011). Patients may also have a mild anemia of chronic disease and modest elevations in the serum immunoglobulins between attacks (Eliakim et al., 1981). A minority of patients have an increased serum IgD, although usually not to the level seen in hyperimmunoglobulinemia D with periodic fever syndrome (Medlej-Hashim et al., 2001).

Laboratory abnormalities are associated with the amyloidosis of FMF. The earliest finding is isolated albuminuria, without hematuria, with a normal or only slightly depressed serum albumin and normal renal function. As proteinuria increases, the serum albumin begins to fall, and renal function deteriorates over approximately 3 to 5 years to the point of renal failure necessitating replacement therapy. During this period hyperphosphatemia, acidosis, hyperkalemia, and anemia evolve, consistent with uremia.

MOLECULAR BASIS OF FMF: THE DISEASE GENE, MEFV

The gene mutated in FMF, *MEFV,* comprises 10 exons over an approximately 15 kb genomic interval on chromosome 16p13.3 (International FMF Consortium, 1997) and encodes a 781 a.a. open reading frame that is expressed in granulocytes, cytokine-activated monocytes, and synovial and peritoneal fibroblasts (Centola et al., 2000; Diaz et al., 2004; Matzner et al., 2000; Papin et al., 2003). However, identification of transmission of FMF following allogeneic bone marrow transplant emphasizes the role of bone marrow-derived cells in disease pathogenesis (Petropoulou et al., 2010; Touitou et al., 2007a). Initial computational analysis of the conceptual protein identified a cassette comprising a

B-box zinc finger (residues 375–407), an α-helical coiled-coil domain (residues 408–594), and a B30.2 domain (residues 598–774), all of which have been implicated in protein–protein interactions.

At the time of this writing, more than 90 FMF-associated *MEFV* mutations have been identified, with over 100 additional mutations and variants of unknown significance. An updated collection of these mutations is available on the Infevers website (http://fmf.igh.cnrs.fr/ISSAID/infevers/; Milhavet et al., 2008).The majority of the mutations are in exon 10, encoding the B30.2 domain, with several "hot spots" (M680, M694, K695, F743) that have more than one known mutation. The second major cluster of mutations is in exon 2, with a mutational "hot spot" at E148.

FUNCTION OF PYRIN, THE MEFV GENE PRODUCT

Work on the function of pyrin, the *MEFV* gene product, has focused on interactions mediated by the N-terminal pyrin domain. Similar to cryopyrin, the PYRIN domain of pyrin interacts specifically with the homologous domain of ASC, forming a pyrin inflammasome (Chae et al., 2003; Dowds et al., 2003; Masumoto et al., 1999, 2003; Richards et al., 2001). These proteins co-localize to polymerizing, actin-rich regions of the cell (Waite et al., 2009). As in the cryopyrinopathies, ASC binding of caspase 1 leads to the cleavage of caspase 1 into its enzymatically active subunits (Agostini et al., 2004; Chae et al., 2003; Martinon et al., 2002, 2004b; Srinivasula et al., 2002; Stehlik et al., 2003; Wang et al., 2002), which results in IL-1β cleavage and activation (Fig. 33.1). The critical role of mutations in pyrin leading to persistent inflammation has been confirmed in a murine knock-in model and further implicates the role of IL-1β in disease pathogenesis in an ASC-dependent manner (Chae et al., 2011).

Although less completely understood, the interaction of pyrin with ASC also appears to regulate leukocyte apoptosis. Peritoneal macrophages from pyrin-deficient mice exhibit a defect in apoptosis through a caspase 8-dependent, IL-1β–independent pathway, suggesting a role for wild-type pyrin in limiting the duration of the innate immune response through cell death (Chae et al., 2003). Nevertheless, underscoring the complexity of the process, in certain transfection systems wild-type pyrin exerts an antiapoptotic effect (Dowds et al., 2003; Masumoto et al., 2003; Richards et al., 2001).

Finally, pyrin may modulate NF-κB activation, another important component of the innate immune response, via two mechanisms: its interaction with ASC and as a direct substrate for caspase 1. ASC has been shown to bind to components of the IκB kinase complex, which regulates NF-κB through the phosphorylation of IκB (Stehlik et al., 2002). Depending on the cellular context, co-transfection of wild-type pyrin with ASC may potentiate or suppress NF-κB activation (Dowds et al., 2003; Masumoto et al., 2003; Stehlik et al., 2002). In a second pathway leading to NF-κB translocation, caspase 1 may cleave pyrin itself, and these cleavage events are notably greater in leukocytes from FMF patients than those from healthy controls (Chae et al., 2008). These data may account for the gain-of-function and autoinflammatory phenotype observed even in single-FMF-mutation carriers.

While the interaction of the N-terminal domain of pyrin with ASC sheds new light on the regulation of inflammation, it does not yet explain the molecular mechanism by which missense mutations in pyrin, many of which are at the C-terminal end of the protein, lead to autoinflammatory disease. Possibly, these mutations indirectly influence the effect of pyrin on IL-1β processing, apoptosis, and/or NF-κB activation, perhaps conferring a selective advantage by pushing the balance, under some circumstances, toward heightened innate immunity.

MUTATION ANALYSIS IN FMF

Prior to the identification of *MEFV,* FMF was thought to be primarily a disease of non-Ashkenazi Jews, Armenians, Arabs, and Turks. Availability of genetic testing has identified numerous cases among several additional populations with Mediterranean roots, including Ashkenazi Jews (Aksentijevich et al., 1999; Gershoni-Baruch et al., 2001; Kogan et al., 2001; Samuels et al., 1998; Stoffman et al., 2000), Italians (Aksentijevich et al., 1999; La Regina et al., 2003; Samuels et al., 1998), Greeks (Konstantopoulos et al., 2003), Spaniards (Touitou, 2001), and Cypriots (Deltas et al., 2002), and in a broad range of other ethnicities (Dode et al., 2000; Touitou, 2001). In some of these newly recognized patients, the clinical phenotype is milder than that of the classically affected ethnic groups, perhaps owing to documented differences in the population distribution of specific *MEFV* mutations.

Direct screening for mutations has confirmed previous estimates of extraordinarily high carrier frequencies in several ethnic groups. Carrier frequencies of at least 20 percent have now been established among North African, Iraqi, and Ashkenazi Jews, Arabs, Turks, and Armenians (Gershoni-Baruch et al., 2001; Kogan et al., 2001; Stoffman et al., 2000; Yilmaz et al., 2001). Since these figures overestimate the disease frequencies (Ozen et al., 1998; Yuval et al., 1995), it is likely that there are substantial numbers of individuals with two *MEFV* mutations but minimal symptoms. Recent family studies support this assertion (Tunca et al., 2002). The high frequencies combined with availability of genetic testing has led to a third subset of patients, phenotype III, described as subclinical or preclinical FMF, in which patients have two *MEFV* mutations but are asymptomatic.

As noted above, over half of the known mutations in *MEFV* are located in exon 10, a figure that is enhanced when the frequencies of the various mutations among patients are taken into account (Touitou, 2001). Among certain populations, such as Ashkenazi Jews, E148Q (exon 2) and P369S (exon 3) are also observed at relatively high frequency (Aksentijevich et al., 1999). For this reason, some commercial and academic laboratories screen for FMF by sequencing exon 10 and performing mutation-specific tests, such as restriction endonuclease assays, for E148Q and P369S.

Identification of specific mutations may have important implications for prognosis, and increased genetic testing has identified a spectrum of disease. E148Q may be the only

identifiable mutation in *trans* with an exon 10 mutation in patients with moderate to severe disease, yet asymptomatic individuals with the same genotype have been found within the same kindreds (Akar et al., 2001; Aksentijevich et al., 1999; Ben-Chetrit et al., 2001a; Booth et al., 2000; Samuels et al., 1998). At the other end of the genotype–phenotype spectrum is M694V. Studies of Jewish, Arab, and Armenian patients indicate that M694V homozygotes are at increased risk of amyloidosis, possibly through reduced responsiveness to colchicine (Brik et al., 1999; Cazeneuve et al., 1999; Gershoni-Baruch et al., 2002; Gershoni-Baruch et al., 2003; Majeed et al., 2002; Mansour et al., 2001), although data in the Turkish population are conflicting (Soylemezoglu et al., 2010; Turkish FMF Study Group, 2005). M694V homozygotes may also be at increased risk for arthritis and erysipeloid erythema, as well as for an early age of onset and frequent attacks (Brik et al., 1999; Cazeneuve et al., 1999; Dewalle et al., 1998; Gershoni-Baruch et al., 2002; Kone Paut et al., 2000). Although the data are less extensive, the M694I mutation may also confer increased risk of amyloidosis (Ben-Chetrit et al., 2001a; Mansour et al., 2001).

Mutational analysis has identified a substantial fraction (up to 30 percent) of patients with typical clinical findings of FMF, including a therapeutic response to colchicine, who have only one demonstrable *MEFV* mutation (Akar et al., 2000; Aksentijevich et al., 1999; Cazeneuve et al., 1999; Mansour et al., 2001; Medlej-Hashim et al., 2000; Padeh et al., 2003), even when the entire coding region is screened (Cazeneuve et al., 2003). It is possible that, at least on certain genetic backgrounds, a single *MEFV* mutation is sufficient to manifest symptoms. Consistent with this view, it is now well established that FMF carriers often exhibit biochemical evidence of intermittent inflammation, and there are at least two rare *MEFV* mutations (ΔM694 and the M694I-E148Q complex allele) that transmit clinically typical FMF in a dominant fashion (Booth et al., 2000). Finally, on the basis of Turkish FMF families unlinked to chromosome 16 (Akarsu et al., 1997) and FMF patients from Palma de Mallorca with no demonstrable *MEFV* mutations (Domingo et al., 2000), some have posited the existence of at least one additional FMF gene besides *MEFV,* perhaps accounting for the "mutation deficit" in symptomatic heterozygotes.

STRATEGIES FOR THE DIAGNOSIS OF FMF

In many instances, the possibility of FMF is first considered in a child with recurrent, unexplained episodes of fever, with or without the localized inflammatory manifestations described above. In Western countries, patients often experience multiple episodes over a period of several months before the diagnosis is entertained, and it is not unusual for such patients to have already undergone extensive evaluations for infection, malignancy, and, in some cases, inflammatory bowel disease. It is important to recall that there are occasional FMF patients without known Mediterranean heritage, and, owing to the recessive mode of inheritance of FMF and the small family size and increased mobility of Western society, about half of the patients in most series have no known family history of periodic fever.

A number of sets of clinical criteria were proposed prior to the identification of *MEFV,* the most widely quoted of which was developed at a large center in Tel-Hashomer, Israel (Livneh et al., 1997b). The various sets of criteria agree that the cardinal features of FMF are short (12 hour to 3 day), recurrent (three or more) episodes of fever (rectal temperature >38°C) with painful manifestations in the abdomen, chest, joints, or skin, in the absence of any other demonstrable causative factors. The Tel-Hashomer criteria enumerate milder attacks, exertional leg pain, and a favorable response to colchicine as minor criteria, and a positive family history, age of onset less than 20 years, appropriate ethnicity, parental consanguinity, an acute phase response during attacks, episodic proteinuria/hematuria, and an unproductive laparotomy as supportive criteria. The diagnosis is then established with appropriate combinations of major, minor, and supportive criteria.

Although the Tel-Hashomer criteria perform extremely well in high-risk populations, they probably do not work as well in Western nations, where the disease is milder (due to a different spectrum of mutations), physicians have had much less clinical experience with FMF, and the frequency of other hereditary periodic fevers may be higher than the frequency of FMF. Clinical features that may help differentiate FMF from the other hereditary periodic fevers include ethnicity, duration of attacks, type of skin rash, and responsiveness to colchicine (Table 33.1).

Genetic testing has assumed a major adjunctive role in the diagnosis of FMF and has extended both the clinical and the ethnic spectrum of FMF. Nevertheless, the aforementioned issues of sensitivity (the "mutation deficit") and penetrance underscore the need to consider clinical information in the interpretation of *MEFV* genetic test results (Booty et al., 2009).

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

Standard teaching is that FMF is inherited as an autosomal recessive disorder with reduced penetrance in females (M:F ratios approximately 1.5 to 1). Since the measured carrier frequencies for Jewish, Arab, Armenian, and Turkish populations are often more than 20 percent, in many cases this translates into a risk of 10 percent or more. However, we now know that some mutations, such as M694V, have greater penetrance than others, such as E148Q; that a few *MEFV* mutations truly show a dominant mode of inheritance; that there is a biochemical inflammatory phenotype associated with the FMF carrier state; and that a substantial number of patients (often with relatively mild symptoms) have only one demonstrable mutation. Although such information complicates genetic counseling, it has been widely enough recognized to deserve inclusion in the information provided to patients and their families.

Given the rare occurrence of FMF phenotype II, in which patients present with amyloidosis as the first manifestation of the disease, the question may be raised whether asymptomatic relatives of FMF patients should be screened for *MEFV* mutations, perhaps with the intention of initiating prophylactic

colchicine in those individuals who test positive for two mutations. In Western nations in which phenotype II is exceedingly rare, where the M694V frequency is low and amyloidosis is uncommon even in patients with FMF, screening of asymptomatic family members is generally not performed, since the discovery of "genetic FMF" may adversely affect the insurability of individuals who may never develop symptoms. There is still no consensus on presymptomatic screening in families with a strong history of amyloidosis, and some would advocate careful follow-up rather than colchicine prophylaxis in asymptomatic family members with two mutations.

Prenatal diagnosis of FMF is feasible but requires careful genetic counseling to appropriately explain decreased penetrance and interpretation of heterozygous genotypes. In addition, given the relative effectiveness of colchicine therapy, prenatal diagnosis raises significant moral and ethical issues regarding the possible termination of pregnancy for what many regard to be a treatable disease.

TREATMENT AND PROGNOSIS

Daily oral colchicine therapy has been established as effective in preventing both the acute attacks of FMF and systemic amyloidosis. Nearly three fourths of adult FMF patients taking 1.2 to 1.8 mg of colchicine per day experience a near-complete remission of their attacks, and over 90 percent demonstrate a marked improvement. Colchicine may cause diarrhea or gastrointestinal upset, but this side effect can be minimized by starting at a low dose and gradually titrating upward, and by dividing the daily dose. In some patients the gastrointestinal effects of colchicine are aggravated by lactose intolerance (Fradkin et al., 1995), and a lactose-free diet may be helpful. Colchicine is safe in children and has been shown to have beneficial effects on height and weight parameters (Zung et al., 2006), but it must be carefully titrated to efficacy and toxicity (Ozkaya et al., 2003; Zemer et al., 1991).

Oral colchicine may also prevent amyloid progression in FMF patients who already have proteinuria due to amyloidosis (Akse-Onal et al., 2010; Livneh et al., 1994b; Oner et al., 2003; Sevoyan et al., 2009; Simsek et al., 2000). The prognosis is best if the serum creatinine is less than 1.5 mg/dL and is adversely affected by tubulointerstitial disease at diagnosis and by noncompliance. Doses of 1.5 mg per day or more are most effective in these patients, as well as in patients who have already undergone renal transplantation (Livneh et al., 1992). Posttransplant colchicine may be even more important in preventing renal allograft interstitial fibrosis, as well as amyloid recurrence, and could account for better graft survival rates (Ozdemir et al., 2006). The combination of colchicine and cyclosporine should be avoided when possible in transplant patients, since cyclosporine inhibits the MDR1 transport system required for hepatic and renal colchicine excretion (Gruberg et al., 1999; Minetti et al., 2003; Simkin et al., 2000; Speeg et al., 1992).

Agents that inhibit or compete for the hepatic cytochrome P450 system component CYP 3A4, such as cimetidine, erythromycin, lovastatin, and grapefruit juice, may increase colchicine blood levels (Ben-Chetrit et al., 1998b). Colchicine toxicity other than diarrhea is rare in patients taking standard doses but can occur in the presence of inhibitors of clearance, in elderly patients with renal insufficiency (Altiparmak et al., 2002; Kuncl et al., 1987), or if colchicine is given intravenously to abort an attack in patients already receiving oral colchicine (Bonnel et al., 2002; Putterman et al., 1991; Wallace et al., 1988).

Most experts advise continuing colchicine in female FMF patients during conception and pregnancy. Previously, performing an amniocentesis during the second trimester was proposed to screen for the slightly increased risk of trisomy 21 (Ben-Chetrit et al., 2003). However, retrospective studies (Ben-Chetrit et al., 2010) and a recent prospective observational comparative cohort study examining 238 colchicine-exposed pregnancies demonstrated no increased risk of cytogenetic anomalies (Diav-Citrin et al., 2010), but the issue remains open for further study. Although small concentrations are present in the breast milk of women taking colchicine, breast-feeding is considered safe (Ben-Chetrit et al., 1996).

Colchicine may prevent the attacks of FMF by multiple mechanisms. Colchicine is concentrated in granulocytes, perhaps because these cells express only low levels of the *MDR1*-encoded P-glycoprotein pump (Ben-Chetrit et al., 1998c, 1998d). Through its interaction with microtubules or other less well-defined mechanisms, colchicine inhibits L-selectin expression on neutrophils (Cronstein et al., 1995), which inhibits neutrophil chemotaxis (Bar-Eli et al., 1981; Dinarello et al., 1976). Furthermore, colchicine inhibits the P2X7 pore formation, which is necessary for ATP-induced inflammasome activation and, consequently, IL-1β release from immune cells (Marques-da-Silva et al., 2011).

The management of patients with FMF who do not respond well to colchicine remains controversial, and several adjunctive approaches have been investigated, including subcutaneous interferon-α (Calguneri et al., 2004a, 2004b; Tunca et al., 1997, 2004; Tweezer-Zaks et al., 2008) and weekly low-dose intravenous colchicine (Lidar et al., 2003). More recently, biological therapies targeting TNF (Erten et al., 2011; Ozgocmen et al., 2011) or IL-1β have shown promising results. Specifically targeting IL-1β, given its underlying role in the pathogenesis of FMF, has demonstrated significant benefit in reducing the frequency and severity of attacks, lowering acute phase reactants, and leading to the resolution of proteinuria ε (Meinzer et al., 2011; Mitroulis et al., 2008, 2011; Ozen et al., 2011). Allogeneic bone marrow transplantation has also been proposed as a treatment for refractory FMF (Milledge et al., 2002), but many experts regard the risk–benefit ratio as unacceptable (Touitou et al., 2003).

In a large majority of patients with FMF, the prognosis on standard colchicine therapy is excellent, allowing for a full range of activities and a normal lifespan. Amyloidosis is the major life-limiting manifestation of FMF, but fortunately the incidence is much reduced with colchicine prophylaxis. Renal transplantation has proven to be effective in patients who take adequate doses of colchicine in the posttransplant period, with graft survival similar to non-amyloidotic controls (Celik et al., 2006). Patients who respond poorly to colchicine are

often very impaired functionally and are a major therapeutic priority.

ANIMAL MODELS

Chae et al. (2003) reported the development of a mouse line expressing a truncated, hypomorphic form of pyrin and an inflammatory phenotype. Consistent with the role of pyrin in IL-1β maturation, peritoneal macrophages from these mice exhibited increased caspase 1 activation and increased IL-1β processing and secretion. Peritoneal macrophages from these animals also exhibited a defect in apoptosis that was independent of IL-1β but associated with impaired caspase 8 cleavage. Low doses of bacterial LPS induced an accentuated body temperature response in homozygous mutant mice, and higher doses induced increased lethality in the homozygous mutants relative to wild type. Moreover, the induction of inflammatory peritoneal exudates by thioglycollate was increased in homozygous mutants relative to heterozygotes or wild-type mice.

To study the pathogenesis of specific mutations in pyrin that lead to FMF, the same group generated various gene insertion or "knock-in" murine models using the frequent mutations M680I, M694V, and V726A. Contrary to the truncated pyrin model described above, these mice display a profound inflammatory phenotype, comparable to that observed in FMF patients (Chae et al., 2011). This model confirms that bone marrow-derived cells, specifically granulocytes, are necessary for the disease phenotype, as observed in in vitro studies and transmission following allogeneic bone marrow transplant from a affected donor in human patients (Petropoulou et al., 2010; Touitou et al., 2007a). The role of IL-1β is further implicated, as crossing knock-in mice with IL-1-receptor-deficient mice led to attenuation of inflammatory disease. Additional crosses revealed that mutant PYRIN mediated the activation of ASC and caspase 1 independently of NLRP3, but the stimulus for activation of PYRIN itself remains unknown. As the increased availability of genetic testing has led to the recognition of patients heterozygous for MEFV mutations, with subclinical or mild phenotypes, this model has important implications for a gain-of-function role of PYRIN mutations (Chae et al., 2011).

TNF RECEPTOR-ASSOCIATED PERIODIC SYNDROME

The name "familial Hibernian fever" (FHF; MIM142680) was first proposed to describe a large family of Irish/Scottish ancestry afflicted with recurrent episodes of fever, abdominal pain, myalgia, and erythematous rash that responded to corticosteroid but not colchicine treatment (Williamson et al., 1982). Fifteen years later, a follow-up report confirmed a dominant mode of inheritance in this family and extended the clinical phenotype to include conjunctivitis, periorbital edema, and systemic AA amyloidosis (McDermott et al., 1997). Several other reports described families with similar dominantly inherited inflammatory symptoms from different ethnic backgrounds (Bergman et al., 1968; Gadallah et al., 1995; Gertz et al., 1987; Hawle et al., 1989; Karenko et al., 1992; Mache et al., 1996; Zaks et al., 1997; Zweers et al., 1993).

In 1998, two independent groups mapped the susceptibility loci for FHF and an Australian (Scottish) variant denoted "familial periodic fever syndrome" to chromosome 12p13 (McDermott et al., 1998; Mulley et al., 1998), suggesting that the same locus was responsible for both syndromes. Candidate gene analysis, the central role of TNF signaling in inflammation, and the observation of reduced levels of the soluble p55 receptor in the serum of several patients with FHF led to the identification of mutations in *TNFRSF1A,* which encodes the 55 kDa receptor for TNF (also known as TNFR1, TNFRSF1A, p55, p60, and CD120a; McDermott et al., 1999). The discovery of mutations in *TNFRSF1A* consolidated these clinical variants into a single nosological entity denoted the TNF receptor-associated periodic syndrome (TRAPS).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF TRAPS

In the largest single group of TRAPS patients reported to date (Hull et al., 2002b), the median age of onset of symptoms was 3 years, ranging from 2 weeks to 53 years of age. Males and females are affected equally. In contrast with FMF, the duration of TRAPS attacks is quite variable (Dode et al., 2002a), ranging from short episodes of 1 to 2 days to month-long flares; in rare cases, patients experience nearly continuous, fluctuating symptoms (McDermott et al., 1997). At the onset of an attack, inflammatory symptoms, such as muscle cramping, abdominal pain, or pleuritic chest pain, may be relatively subtle, gradually increasing over the course of 1 to 3 days. Pain often persists at its maximum intensity for several days before gradual resolution. The onset of attacks has been described with physical or emotional stress, menses, or local trauma, but in many cases there does not appear to be a definite provoking stimulus. In women, pregnancy is sometimes associated with remission (Kriegel et al., 2003; McDermott et al., 1997), with possible exacerbation during the postpartum period (Rosen-Wolff et al., 2001).

Nearly all patients develop fever in association with their attacks, although it may be absent during some attacks in adults. Temperature greater than 38°C (maximally 41°C) usually lasts for more than 3 days, often heralding the onset of other inflammatory symptoms.

Serosal involvement is a prominent feature of TRAPS (Hull et al., 2002b). Attacks consisting solely of fever and abdominal pain occur in over 90 percent of patients with TRAPS and can be the result of inflammation of the peritoneum, the abdominal musculature, or both. Serosal inflammation often produces the physical findings of an acute abdomen, and approximately half of the patients in one series had undergone at least one laparoscopy or exploratory laparotomy. Findings frequently include mononuclear infiltrates in the bowel wall or peritoneal adhesions, which eventually can cause bowel necrosis. Pleuritic attacks, with or without radiographic evidence of effusion, occur in about 50 percent of TRAPS patients. Less

common serosal manifestations include pericarditis and episodes of scrotal pain.

Localized myalgia is extremely common during TRAPS attacks. The myalgia of TRAPS manifests as cramplike discomfort, fluctuating in severity but disabling at its worst. Affected areas are warm, tender to palpation, and often associated with an erythematous patch. When found on an extremity, the myalgia and rash of TRAPS characteristically migrate centrifugally (as opposed to spreading) from proximal to distal limb over several days. As the region of inflammation passes over a joint (e.g., knee or elbow), there is often evidence of synovitis and effusion, with transient contracture. Although most commonly affecting the limbs and torso, the myalgia and rash of TRAPS can also occur on the face and neck. During the episodes of myalgia, serum creatine kinase and aldolase concentrations remain normal. MRI demonstrates focal areas of edema in the affected muscular compartments and intramuscular septa (Hull et al., 2002d), and this may persist between episodes (Quillinan et al., 2010). The limited number of biopsies reported in TRAPS patients with active myalgia have demonstrated monocytic fasciitis or lymphocytic vasculitis, but not myositis (Drewe et al., 2003a; Hull et al., 2002d).

TRAPS attacks frequently lead to arthralgia or arthritis, either as a part of the migratory myalgia picture or independently. Polyarticular arthralgia is by far the most common rheumatic manifestation of TRAPS. The arthritis of TRAPS tends to be nonerosive and either monoarticular or pauciarticular and asymmetrical, most commonly affecting the hips, knees, and ankles.

The most distinctive of the cutaneous manifestations of TRAPS is the aforementioned centrifugally migratory erythematous rash (Plate 33.III), sometimes overlying areas of myalgia (Toro et al., 2000). Such lesions are warm and tender, blanch on palpation, and may reach 30 cm in diameter. Histologically, there is a superficial and deep perivascular and interstitial mononuclear cell infiltrate, sometimes with a low-grade lymphocytic vasculitis (Toro et al., 2000). Other less unusual rashes include urticaria-like plaques and serpiginous erythematous patches and plaques (Toro et al., 2000). These variants often involve several different areas of the body simultaneously and are neither migratory nor associated with concurrent myalgia. Relapsing episodes of panniculitis have also been reported in TRAPS (Lamprecht et al., 2004).

Eye involvement occurs in over 75 percent of patients (Hull et al., 2002b). The most frequent ocular findings are conjunctivitis and periorbital edema. Uveitis is relatively rare but should be considered in patients experiencing blurred vision or eye pain.

As in other fever disorders, systemic amyloidosis is the most serious complication of TRAPS (Dode et al., 2002b; Jadoul et al., 2001; Simon et al., 2001b). The frequency of amyloidosis among reported cases is currently about 10 percent. Nephrotic syndrome and renal failure are the most common consequences; there is also one case of hepatic transplantation due to TRAPS amyloidosis reported in the literature (Hull et al., 2002b).

LABORATORY FINDINGS IN TRAPS

The attacks of TRAPS are nearly always associated with a vigorous acute phase response (Hull et al., 2002b), including an accelerated ESR and increased CRP, haptoglobin, fibrinogen, C3 and C4 complement, and ferritin. Although not widely available, the SAA level represents the most sensitive and rapid measure of the acute phase response and may be an important determinant to monitor in patients with systemic amyloidosis (Gillmore et al., 2001). During attacks patients may have prominent leukocytosis and neutrophilia, sometimes with white counts above 20,000/mm^3, and children often have thrombocytosis.

TRAPS patients frequently manifest biochemical evidence of ongoing inflammation even between their acute episodes, although the magnitude of laboratory abnormalities may be diminished. The anemia of chronic disease is common among patients with frequent attacks, and a moderate polyclonal gammopathy is often seen. Although usually normal, modest elevations in the serum IgD have been reported (Dode et al., 2002a; Simon et al., 2001c). Low-titer anticardiolipin antibodies and antinuclear antibodies are seen in a minority of patients.

Albuminuria, usually without significant hematuria, is the most common manifestation of systemic amyloidosis in TRAPS. Initially, renal function is normal, although TRAPS amyloidosis may progress to renal failure within 1 to 2 years. Hepatic involvement usually causes an elevated alkaline phosphatase with normal transaminases.

MOLECULAR BASIS OF TRAPS: THE DISEASE GENE, TNFRSF1A

TNFRSF1A is a 10-exon gene with a 455 codon open reading frame (Fuchs et al., 1992; Loetscher et al., 1990). The encoded protein has a 29 a.a. leader, a 182 a.a. extracellular domain, a 21 a.a. transmembrane domain, and a 223 residue intracellular domain. At the time of this writing, more than 65 mutations in *TNFRSF1A* have been reported in patients with recurrent episodes of fever and inflammation, and an updated database of mutations is available online at the Infevers website (http://fmf.igh.cnrs.fr/infevers/; Milhavet et al., 2008). There are several mutational "hot spots," including five at cysteine residues (C30, C33, C52, C70, and C88, numbered relative to the leucine immediately following the cleavage of the signal peptide) and four noncysteine residues (Y20, H22, T50, and R92). To date, no patients have been identified with mutations in the transmembrane or intracellular domains of the TNFRSF1A protein; null mutations; or mutations in the p75 TNF receptor (TNFRSF1B). Several reports have described families or individuals with TRAPS-like clinical findings but no demonstrable mutations in *TNFRSF1A*, raising the possibility of locus heterogeneity (Aganna et al., 2003; Aksentijevich et al., 2001; Dode et al., 2002a).

FUNCTION OF THE P55 TNF RECEPTOR, THE TNFRSF1A GENE PRODUCT

TNF is a proinflammatory cytokine with pleiotropic biological effects (Vassalli, 1992). At a cellular level, TNF stimulation

can lead either to activation of various signaling pathways (NF-κB or JNK) or apoptosis. As noted above, there are two cellular receptors for TNF, a 55 kDa protein encoded on chromosome 12p13 (TNFRSF1A) and a 75 kDa receptor encoded on chromosome 1p36 (TNFRSF1B) (Smith et al., 1990). Both are members of a family of cell-surface receptors with repeating cysteine-rich extracellular domains (CRDs) (Bazzoni et al., 1996). Both receptors have four extracellular CRDs with intrachain disulfide bonds stabilizing the structure of each CRD. The intracellular segment of the p55 receptor also includes an death domain that is involved in signal transduction (Hsu et al., 1995). The p55 receptor is expressed widely, while the p75 receptor is expressed predominantly on leukocytes and endothelial cells.

TNF forms homo-trimers that, in turn, aggregate homotrimers of either p55 or p75 receptors on the cell surface (Engelmann et al., 1990). All of the cysteine mutations as well as some other mutations (T50M) in the p55 receptor are predicted to have a major effect on its three-dimensional structure, since they would prevent the formation of highly conserved disulfide bonds important to maintaining proper folding of the molecule (Rebelo et al., 2006). Initially, evidence was presented that mutant p55 receptors are refractory to activation-induced cleavage of the ectodomain, a homeostatic process that normally limits repeated signaling at the cell surface and creates a pool of potentially antagonistic soluble receptors (Fig. 33.2). The so-called "shedding" hypothesis was supported by diminished levels of soluble p55 in the serum of several TRAPS patients and increased membrane p55 and reduced activation-induced clearance on peripheral blood leukocytes from patients with one mutation (C52F). Impaired receptor shedding has been observed by flow cytometry in leukocytes from patients with nine mutations (H22Y, C30S, C33G, P46L, T50M, T50K, C52F, F112I, and I170N) (Aganna et al., 2003; Aksentijevich et al., 2001; McDermott et al., 1999; Nevala et al., 2002). The mechanism by which specific disease-associated mutations impair cleavage most likely represents an indirect structural effect, since all but one of the known mutations are remote from the metalloprotease-induced cleavage site (Gullberg et al., 1992).

Current evidence suggests that impaired ectodomain cleavage is unlikely to be the primary explanation for the TRAPS phenotype, since shedding appears to be normal in many patients (Aganna et al., 2003; Aksentijevich et al., 2001; Huggins et al., 2004). Disease-associated mutations have been shown to affect receptor folding and trafficking, resulting in retention of the receptor in the endoplasmic reticulum, loss of normal TNF signaling function, inability to interact with normal TNF receptors, and reduced turnover of mutant TNF receptors (Lobito et al., 2006; Simon et al., 2010; Todd et al., 2004). Intracellular accumulation of the mutant receptor has been shown to activate various signaling pathways, resulting in cytokine-mediated inflammation (Simon et al., 2010). This is supported by the finding of upregulation of several cytokine pathways, altered NF-κB signaling, and defective apoptosis pathways in many patients (D'Osualdo et al., 2006; Nedjai et al., 2008, 2009, 2011; Rebelo et al., 2009; Siebert et al., 2005; Simon et al., 2010; Stjernberg-Salmela et al., 2010), but some of these changes may vary with the specific mutation (Rebelo et al., 2009). In addition, TRAPS patient cells are hyperresponsive to innate immune signals (Simon et al., 2010) and exhibit altered mitochondrial function with enhanced oxidative capacity due to mitochondrial reactive oxygen species generation (Bulua et al., 2011) (Fig. 33.2).

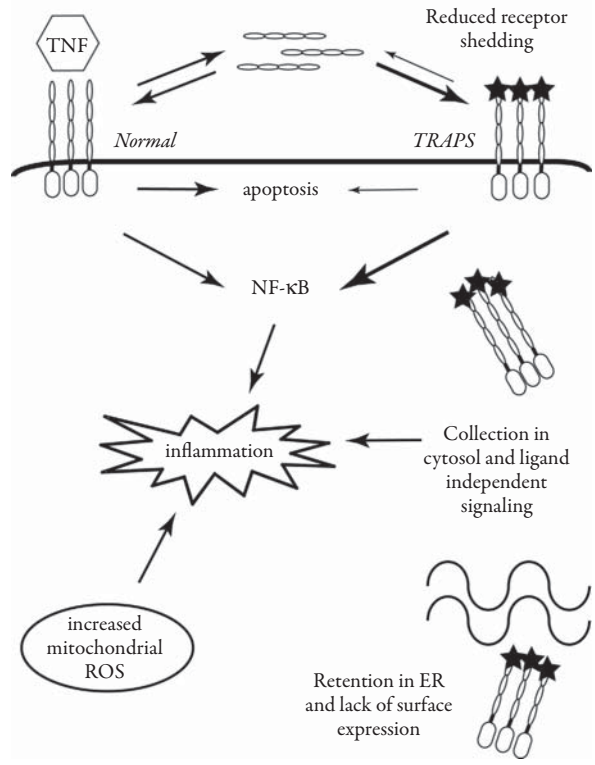

Figure 33.2 Summary of proposed mechanisms of pathogenesis in TNF receptor-associated periodic syndrome (TRAPS). Mutations in the p55 TNF receptor may have several effects on intracellular trafficking, receptor expression, and subsequent signaling following binding of TNF. Reduced metalloprotease cleavage may lead to impaired shedding of sTNFRSF1A and decreased TNF antagonist activity. Mutations affecting surface expression of the TNF receptor may result from structural misconformation with retention of receptor subunits in the endoplasmic reticulum (ER) or collection of p55 in the cytosol with signaling independent of TNF-binding, amplified by enhanced ROS production. Stars denote the presence of mutations.

MUTATION ANALYSIS IN TRAPS

Almost all of the mutations are in exons 2 to 4 of the 10 exon gene and are in the first two cysteine-rich domains (those furthest from the membrane) of the receptor protein. Therefore, traditional mutational screening has focused on these high-probability regions of the gene. The conventional system for numbering mutations in TRAPS found in this chapter and most published reports denotes the leucine that immediately follows the cleavage of the signal peptide as residue 1. Inclusion of the signal peptide would increase these numbers by 29.

There is some genotype–phenotype correlation in that cysteine mutations are often associated with more severe disease and a higher risk of amyloidosis, which may be due to different functional effects of mutations (Rebelo et al.,

2006). However, significant variation in severity has been observed within patients with the same mutation, even within the same family (Lehmann et al., 2010). Furthermore, two specific noncysteine *TNFRSF1A* mutations are sufficiently common to be considered polymorphisms in some populations (Aksentijevich et al., 2001; Ravet et al., 2006). R92Q, which is present in almost half of the independent TRAPS chromosomes identified in a large cohort followed at the U.S. National Institutes of Health, is also present in approximately 1 percent of Caucasian control chromosomes. Since the population frequency of TRAPS is far less than 1 percent, the penetrance of this variant must be low. However, data are accumulating that the TRAPS phenotype is but one end of a spectrum of inflammatory conditions associated with R92Q (Cantarini et al., 2009; Pelagatti et al., 2011). Similarly, the P46L variant is found in approximately 2 percent of African-American control chromosomes (Aksentijevich et al., 2001) and at an even higher frequency among some sub-Saharan African populations (Tchernitchko et al., 2004).

STRATEGIES FOR THE DIAGNOSIS OF TRAPS

Clinical suspicion is the cornerstone of the diagnosis of TRAPS. Among the clinical features of TRAPS that help distinguish it from the other periodic fever syndromes are the duration of attacks (episodes lasting >1 week suggest TRAPS, although shorter attacks can be seen), the migratory myalgia and overlying erythematous rash, the presence of periorbital edema during attacks, a dominant pattern of inheritance, and the differential response to corticosteroids over colchicine (Table 33.1). Systemic-onset juvenile idiopathic arthritis (SOJIA) and adult-onset Still's disease can be distinguished by the fever pattern (temperatures usually return to normal or below normal each day), the clinical course of the arthritis (progressive, erosive polyarticular arthritis), and the cutaneous manifestations (evanescent salmon-colored macular or maculopapular rash).

Once the clinical suspicion has been established, the diagnosis of TRAPS may be confirmed by *TNFRSF1A* sequence analysis. Most diagnostic laboratories routinely screen exons 2 to 5 of the gene, which would detect most of the currently known mutations. More complete sequencing may be entertained if the clinical suspicion is high, but the yield is relatively low. As in FMF and the cryopyrinopathies, there are numerous sporadic and some familial cases that clinically resemble TRAPS but do not have *TNFRSF1A* mutations, suggesting involvement of additional genes (Cantarini et al., 2012).

Serum levels of soluble p55 receptor, while of theoretical interest, have not proven to be an adequate substitute for genetic testing since they can be low, normal, or high among patients who are suspected of having TRAPS but are found to be mutation negative (Aganna et al., 2003). Moreover, although some mutation-positive TRAPS patients do have low levels of soluble p55, inflammatory attacks or renal insufficiency can spuriously normalize soluble p55 measurements.

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

Although TRAPS is inherited in an autosomal dominant fashion, penetrance is not 100 percent, even for substitutions at cysteine residues. Further complicating the issue, several cases of de novo *TNFRSF1A* mutations have been reported (Aganna et al., 2002b, 2003). Careful history taking can sometimes identify unsuspected cases among the allegedly unaffected relatives of TRAPS patients, and genetic testing is of benefit in establishing the correct diagnosis in these individuals. Genetic testing may also be warranted for asymptomatic relatives in families with a strong history of TRAPS-associated amyloidosis. However, in cases where the risk of amyloidosis is low, the possible benefits of identifying other mutation-positive asymptomatic relatives should be weighed against the potential impact of such information on insurability. Similarly, the use of molecular techniques for prenatal diagnosis should be approached with great caution, since there are still insufficient data to make reliable estimates of penetrance, and effective, targeted cytokine-inhibitory therapies are now available.

TREATMENT AND PROGNOSIS

In contrast with FMF, colchicine prevents neither the acute attacks nor the amyloidosis of TRAPS (Dode et al., 2002b; Hull et al., 2002b). The efficacy of nonsteroidal anti-inflammatory drugs is limited to mild attacks. Short courses of oral or parenteral corticosteroids may be effective in more severe episodes, but escalating doses are often required, with the attendant toxicities. Initial studies demonstrated that twice- or thrice-weekly administration of etanercept, the p75 TNFR:Fc fusion protein, was effective in reducing, although usually not eliminating, the clinical and laboratory manifestations of TRAPS and reducing corticosteroid use (Drewe et al., 2003b; Galon et al., 2000; Hull et al., 2002a, 2002b; Kastner et al., 1999; Lamprecht et al., 2004; Nigrovic et al., 2001; Simon et al., 2001c; Weyhreter et al., 2003). Etanercept may also have a role in preventing amyloid deposition, although monitoring of SAA levels is necessary to titrate the dosage (Drewe et al., 2000, 2004; Hull et al., 2002c). However, the response is highly variable and positive effects may not be sustained, as there are reports of patients with progressive amyloidosis while on etanercept therapy (Hull et al., 2002c; Simsek et al., 2010). Other anti-TNF antibody therapies, such as infliximab, have either been ineffective or have had a paradoxical response with worsening symptoms (Bodar et al., 2007a; Drewe et al., 2007; Jacobelli et al., 2007; Nedjai et al., 2009).

More recently, the use of other non-TNF biologics has shown promise in the treatment of TRAPS. A number of case reports and two small series have demonstrated the efficacy and safety of anakinra in patients with TRAPS who had persistent symptoms despite treatment with daily prednisone or with etanercept. Daily subcutaneous anakinra eliminated symptoms and led to normalization of CRP and SAA and to the resolution of proteinuria with stabilization of renal function (Gattorno et al., 2008; Obici et al., 2011; Sacre et al., 2008; Simon et al., 2004a). Additionally, a preliminary report

of one patient with TRAPS demonstrated that treatment with tocilizumab (IL-6 antibody) aborted an evolving attack and prevented further attacks of TRAPS during the therapeutic period and reduced acute phase reactants (Vaitla et al., 2011). These early studies suggest that biological modulation of other inflammatory cytokines may be effective treatment options for patients with refractory symptoms, and are consistent with the role of multiple cytokine pathways in the pathogenesis of TRAPS.

The prognosis of TRAPS is largely related to development of systemic amyloidosis, which is more frequent among patients with cysteine substitutions or a positive family history of amyloidosis (Aksentijevich et al., 2001; Kallinich et al., 2004). Infrequently, TRAPS patients develop life-threatening abdominal complications, such as bowel obstruction, abscesses, or necrosis.

ANIMAL MODELS

Kollias et al. reported knock-in mice that express a nonsheddable p55 TNF receptor with a mutation at the cleavage site (Xanthoulea et al., 2004). These mice exhibited a dominantly inherited autoinflammatory phenotype that supports the hypothesis that TNF receptor shedding is an important negative homeostatic mechanism. Mutant mice developed chronic active hepatitis, were more susceptible to the toxic effects of bacterial LPS and TNF, and exhibited increased susceptibility to experimental autoimmune encephalomyelitis and TNF-induced arthritis. Moreover, macrophages from these mice exhibited increased innate immune responses to Toll-like receptor stimulation.

Recently, knock-in mice with disease-associated missense mutations were reported that confirmed functional studies in humans supporting intracellular accumulation of mutant receptors and enhanced mitochondrial oxidative capacity. Heterozygous mutant mice were hypersensitive to LPS-induced shock while homozygous mice were resistant, similar to TNFR1 knockout mice, suggesting functional cooperation of WT and mutant receptors in TRAPS consistent with an autosomal dominant model. In addition, pharmacological blockade of mitochondrial reactive oxygen species (ROS) reduced the inherent hyperresponsiveness in these mice, emphasizing that multiple intracellular signaling pathways may contribute to the inflammatory attacks in TRAPS (Bulua et al., 2011; Simon et al., 2010).

HYPERIMMUNOGLOBULINEMIA D WITH PERIODIC FEVER SYNDROME

Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS, MIM 260920) was initially described as a unique disorder in 1984, in six Dutch patients with a clinical picture similar to FMF but distinguishing features of prominent lymphadenopathy and less severe abdominal symptoms than usually seen with FMF, as well as their Dutch ancestry (without relatives of the typical ethnicities for FMF) (van der Meer et al., 1984). Moreover, all six Dutch patients were found to have polyclonal elevations in their serum IgD levels, and all five of the Dutch patients who underwent bone marrow aspiration and biopsy had markedly increased numbers of δ^+ plasma cells in the marrow. The HIDS nomenclature was proposed to describe this apparently new periodic fever syndrome as a clinical entity (Drenth et al., 1994b).

Three of the initial patients had a positive family history, but it was only after additional patients and families were identified that a clear autosomal recessive mode of inheritance was apparent (Drenth et al., 1994c; Livneh et al., 1997a). In 1999, 15 years after its initial description, two groups from the Netherlands independently discovered HIDS-associated mutations in *MVK,* the gene encoding mevalonate kinase, an enzyme involved in cholesterol and nonsterol isoprene biosynthesis (Drenth et al., 1999; Houten et al., 1999). Although not well understood, *MVK* mutations still account for most cases of clinical HIDS (Simon et al., 2001a). Nevertheless, as is the case for both FMF and TRAPS, elucidation of the underlying gene has permitted recognition of additional complexity. Thus, it is now clear that some periodic fever patients have raised serum IgD levels without *MVK* mutations (Simon et al., 2001a), while others with *MVK* mutations have normal IgD levels (Frenkel et al., 2000; Houten et al., 1999; Saulsbury, 2003; Takada et al., 2003). As we gain further insight into the role of the mevalonate pathway in regulating inflammation, the study of HIDS promises dividends that will extend far beyond these patients.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF HIDS

HIDS was first recognized in the Netherlands, and even 20 years later the majority of reported cases are of Dutch or neighboring northern European ancestry (Drenth et al., 2001a; Simon et al., 2003). Typically, febrile attacks begin within the first year of life, often precipitated by childhood immunizations, and there is no gender bias (Drenth et al., 1994b). The duration of attacks, 3 to 7 days, is somewhat longer than the duration of FMF attacks but shorter than the episodes sometimes seen in TRAPS. In addition to immunizations, minor infections, trauma, surgery, and menses may trigger attacks. On average attacks occur about once or twice a month, but usually without true periodicity, in childhood and adolescence. Attacks may become less frequent or severe in adults, with the majority of patients reporting more attacks prior to the age of 20 years and fewer attacks after the age of 20 years (van der Hilst et al., 2008a).

The attacks of HIDS often begin with chills and headache. In children, diffuse tender lymphadenopathy, particularly in the cervical chains, is common and is much more frequent than in FMF or TRAPS. As in the latter two disorders, abdominal pain is often present during attacks. Although the incidence of peritoneal signs in HIDS is much lower than in FMF or TRAPS (Drenth et al., 1994b; Livneh et al., 1997a), some patients may still present with an acute abdomen, prompting suspicion for appendicitis or mimicking inflammatory bowel disease and resulting in exploratory surgery (Oretti et al.,

2006; van der Hilst et al., 2008a). Moreover, whereas the abdominal attacks of FMF and TRAPS are frequently associated with constipation, because of decreased peristalsis, the abdominal attacks of HIDS are often accompanied by diarrhea and vomiting. Nevertheless, nearly 10 percent of patients with HIDS do develop adhesions, probably due to recurrent, sterile, peritoneal inflammation (van der Hilst et al., 2008a). Scrotal pain (Saulsbury, 2003) and pericarditis (Breda et al., 2009) have been reported in HIDS, but pleurisy has not been observed.

HIDS can present with a number of different mucocutaneous findings, including diffuse, painful, erythematous macules, a diffuse, erythematous macular and papular rash (Plate 33.IV), erythematous papules and nodules, urticaria, and a morbilliform rash (Drenth et al., 1994a, 1994b). Unlike FMF, there is no predilection for the feet, ankles, or lower legs, and, unlike TRAPS, the rash of HIDS is not migratory. Histologically, cutaneous vasculitis, perivascular inflammatory cells, and deposits of IgD, IgM, or C3 may be present. Other forms of cutaneous vasculitis reported in HIDS include Henoch-Schönlein purpura (Haraldsson et al., 1992) and erythema elevatum diutinum (Miyagawa et al., 1993). Aphthous ulcers of the mouth and vagina may also be seen in HIDS, which, in combination with fever and cervical lymphadenopathy, may be confused with the syndrome of periodic fever, aphthous stomatitis, pharyngitis, and adenopathy (see below) (Marshall et al., 1987, 1989; Thomas et al., 1999).

Joint manifestations are the third most common symptom of HIDS, after abdominal pain and lymphadenopathy, and are most frequently reported as polyarthralgia (Drenth et al., 1993, 1994b; Haraldsson et al., 1992; Loeliger et al., 1993). HIDS may also present with intermittent episodes of polyarticular arthritis of the large joints, usually but not always with fever, and sometimes coinciding with abdominal attacks. Arthritic attacks are more common in children and are usually nondestructive, although joint contractures have been reported in four patients with HIDS (van der Hilst et al., 2008a). As in FMF, the sterile synovial fluid is rich in granulocytes.

Ocular symptoms tend to be limited to the more severe cases of mevalonic aciduria, consisting of retinal dystrophy, optic atrophy, and cataracts. Until recently, no cases of ocular inflammation had been reported in HIDS.

Systemic amyloidosis is uncommon in HIDS but has been reported (D'Osualdo et al., 2004; Obici et al., 2004; Siewert et al., 2006; Tsimaratos et al., 1999) with an estimated frequency of less than 3 percent of patients (van der Hilst et al., 2008b). Despite elevated levels of SAA, both during and between attacks, there is no progression to amyloidosis in most patients with HIDS (van der Hilst et al., 2005). This was previously postulated to be secondary to the milder phenotype, or resolution of the inflammatory episodes in adulthood. However, in vitro human cell culture studies of the HMG-CoA reductase inhibitor lovastatin as a model for the deficiency of isoprenoid products as seen in MVK deficiency have demonstrated that the isoprenoid pathway may play a critical role in the development of amyloid deposits (van der Hilst et al., 2008b). This suggests that mutations in MVK may offer some level of protection against amyloid-induced end-organ disease and, perhaps more importantly, identifies statins as a potential therapeutic agent for patients with autoinflammatory disorders that are susceptible to amyloidogenesis (van der Hilst et al., 2008b).

LABORATORY FINDINGS IN HIDS

Prior to the identification of the underlying gene, HIDS was defined by the polyclonal elevation of the serum IgD (>100 IU/mL or >14.1 mg/dL) on two occasions at least 1 month apart (Drenth et al., 1994b). While the vast majority of *MVK* mutation-positive patients with recurrent fevers meet this criterion, a small percentage of these patients do not. Serum IgD levels do not correlate with the severity of HIDS, either when following an individual patient or when comparing patients, and do not predictably fluctuate with attacks (Drenth et al., 1994b; Hiemstra et al., 1989). Over 80 percent of HIDS patients also have persistent polyclonal increases in serum IgA levels, the significance of which remains unclear (Haraldsson et al., 1992; Hiemstra et al., 1989; Klasen et al., 2001). Although it has been postulated that mevalonate pathway products modulate the induction or survival of IgD-producing B cells (Chen et al., 2011), the true mechanism by which mevalonate kinase deficiency influences IgD levels remains unknown.

As in FMF and TRAPS, during their inflammatory attacks HIDS patients present with leukocytosis and a left shift, an accelerated ESR, elevated acute phase reactants (including CRP and SAA), and occasionally transient hematuria (Drenth et al., 1994b, 2001a; Frenkel et al., 2000). Serum levels of several proinflammatory cytokines may be increased during attacks, as is the urinary excretion of neopterin and leukotriene E_4 (Drenth et al., 1995; Frenkel et al., 2001b). Urinary levels of mevalonic acid are also markedly increased during attacks (Drenth et al., 1999; Frenkel et al., 2001a; Houten et al., 1999; Kelley et al., 2002).

MOLECULAR BASIS OF HIDS: THE DISEASE GENE, MVK

HIDS is caused by recessively inherited mutations in *MVK*, encoded on the long arm of human chromosome 12 (Drenth et al., 1999; Houten et al., 1999). HIDS-associated mutations lead to a marked reduction in mevalonate kinase enzymatic activity (discussed below), but fibroblasts from patients nevertheless typically demonstrate 1 to 3 percent of the activity seen in fibroblasts from healthy controls. *MVK* mutations leading to the complete loss of enzymatic activity cause mevalonic aciduria, an extremely rare condition that manifests not only the periodic fevers, lymphadenopathy, abdominal pain, rash, and arthralgia seen in HIDS, but also dysmorphic features, hypotonia, mental retardation, cataracts, and failure to thrive, which result in a compromised life expectancy (Hoffmann et al., 1986, 1993; Kelley, 2000).

MVK spans a genomic region of 22 kb and comprises 11 exons, the first of which encodes most of the 5′ untranslated region in the cDNA (Cuisset et al., 2001; Houten et al., 2001). The gene encodes a 396 a.a. protein (Schafer et al.,

1992). At the time of this writing, more than 100 HIDS-associated mutations have been identified, and an updated database of mutations is available at the Infevers website (http://fmf.igh.cnrs.fr/ISSAID/infevers/; Milhavet et al., 2008). HIDS-associated *MVK* mutations are distributed throughout the coding sequence predominantly affecting stability and folding of the mevalonate kinase protein (Mandey et al., 2006), while mutations causing mevalonic aciduria are concentrated around sequences encoding the active sites of the mevalonate kinase enzyme (Cuisset et al., 2001; Houten et al., 2001).

FUNCTION OF MEVALONATE KINASE, THE MVK GENE PRODUCT

Mevalonate kinase catalyzes the conversion of mevalonic acid to 5-phosphomevalonic acid in the synthesis of the sterols, which include cholesterol, vitamin D, bile acids, and steroid hormones (Brown et al., 1997; Goldstein et al., 1990; Valle, 1999). This pathway also leads to the production of nonsterol isoprenoids, which are involved in a host of cellular functions. It is unlikely that the autoinflammatory phenotype of HIDS is due to a deficiency in cholesterol, since HIDS patients usually have cholesterol levels in the low-normal range, and since the autoinflammatory phenotype is not seen in patients with other more profound disorders of the mevalonate pathway, such as Smith-Lemli-Opitz syndrome, in which cholesterol deficiency is more significant (Kelley, 2000).

There are several hypotheses on the pathogenesis of HIDS (Fig. 33.3). The first proposes that the inflammatory phenotype is due to the buildup of mevalonic acid, the substrate for the mevalonate kinase enzyme (Simon et al., 2004b). The second hypothesis holds that the disorder is due to deficiencies in isoprenoids normally synthesized through the mevalonate pathway (Frenkel et al., 2002). Although there are in vitro data demonstrating accentuated IL-1β secretion in HIDS leukocytes that can be reversed by the addition of farnesol and geranyl-geraniol (isoprenoid compounds), the issue has been controversial. More recently, the role of apoptosis in HIDS pathogenesis has been proposed in one study noting decreased apoptosis in stimulated lymphocytes from HIDS patients during a symptom-free interval.

The association of HIDS attacks with immunizations and infections may be accounted for by these hypotheses. The decreased apoptosis of stimulated lymphocytes suggests failure to curtail the immunological response, with subsequent systemic inflammation after a minor stimulus (Bodar et al., 2007b). Both the isoprenoid deficiency and mevalonate accumulation hypotheses predict a worsening of symptoms with decreased mevalonate kinase enzymatic activity. In vitro studies of cell lines harboring wild-type or HIDS-mutant *MVK* indicate that the mutant enzyme functions best at 30°C, with a diminution at 37°C and further decreases at 39°C (Houten et al., 2002). This temperature-specific response supports the association of HIDS attacks with immunizations and infections and may also account for the increased urinary mevalonic acid levels seen during HIDS attacks. The role of fever on the apoptotic pathway requires evaluation.

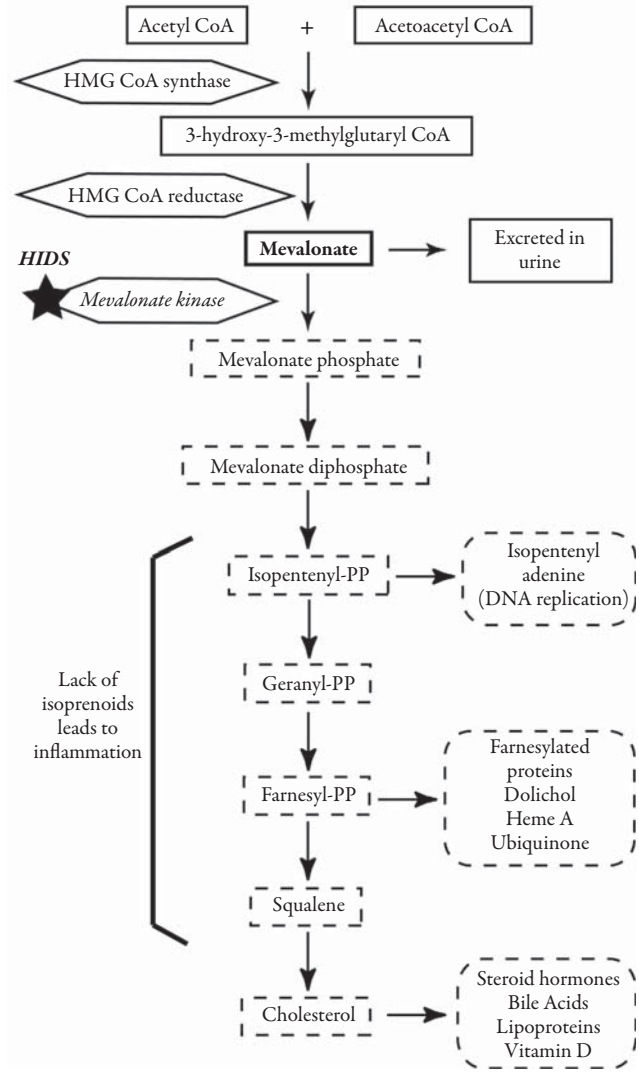

Figure 33.3 Interruption of the mevalonate pathway in the hyperimmunoglobulinemia D with periodic fever syndrome (HIDS). HIDS is caused by recessively inherited mutations in the mevalonate kinase enzyme. HIDS-associated mevalonate kinase mutations typically leave 1 to 3 percent residual enzymatic activity, but the buildup of the mevalonate kinase substrate, mevalonic acid, may be detected in the urine of HIDS patients during inflammatory episodes. Failure of the mevalonate pathway to progress results in decreased isoprenoid formation and subsequent inflammation. Stars denote the presence of mutations. PP, pyrophosphate.

MUTATION ANALYSIS IN HIDS

A very high percentage of HIDS patients harboring *MVK* mutations have at least one copy of the substitution of isoleucine for valine at residue 377 (V377I) (Cuisset et al., 2001; D'Osualdo et al., 2004; Houten et al., 2001). Population-based surveys of newborns in the Netherlands indicate a carrier frequency of 0.6 percent for the V377I mutation among the Dutch (Houten et al., 2003), with haplotype data supporting a founder effect (Houten et al., 2003). Analysis of *MVK* genotypes among HIDS patients indicate an underrepresentation of V377I homozygotes, suggesting a milder phenotype or reduced penetrance for V377I homozygotes (Houten et al., 2003).

Because many patients with HIDS are heterozygous for V377I, commercial and academic laboratories may perform an initial screen for the V377I mutation by restriction endonuclease analysis, and occasionally for the second most common mutation I268T. More extensive screening, usually by DNA sequencing, is reserved for patients who are heterozygous for either mutation (but not for compound heterozygotes, in whom the molecular diagnosis of HIDS would be already established).

STRATEGIES FOR THE DIAGNOSIS OF HIDS

There are currently three possible strategies for diagnosing HIDS: clinical, molecular, and biochemical. Consistent with practice before the identification of the underlying gene, the clinical diagnosis of HIDS is established by documenting elevations in serum IgD levels (>100 IU/mL, or >14.1 mg/dL) on two occasions at least a month apart, with a compatible clinical history. The molecular diagnosis is established by documenting two mutations in *MVK*, while the biochemical diagnosis is established by documenting elevations in urinary mevalonic acid during attacks, or decreased enzymatic activity in cells cultured from the patient. In many cases, all three strategies agree, in which case the patient is sometimes said to have "classic-type HIDS" (Simon et al., 2001a).

Although there is a strong concordance between molecular and biochemical diagnosis, clinical and molecular diagnoses sometimes diverge. About one quarter of patients satisfying clinical criteria for HIDS have no demonstrable *MVK* mutations (Simon et al., 2001a). These patients tend to have milder disease and lower IgD levels than patients with classic-type HIDS and in the past have been called "variant-type HIDS." There are also a few patients who meet genetic and biochemical criteria for HIDS, with a compatible clinical picture, except for persistently normal IgD levels (Frenkel et al., 2000; Houten et al., 1999; Saulsbury, 2003; Takada et al., 2003), although the sensitivity of a high IgD has been estimated at only 0.79 (Ammouri et al., 2007). This discrepancy is more frequent in young children, who may eventually exhibit increased serum IgD levels (Drenth et al., 2001a).

Further complicating the picture, a small percentage of patients with FMF and TRAPS may have modest elevations in their serum IgD, although not to the very high levels seen in some HIDS patients (Dode et al., 2002a; Livneh et al., 1997a; Medlej-Hashim et al., 2001; Simon et al., 2001c). The differential diagnosis of an elevated serum IgD level also includes IgD multiple myeloma, Hodgkin's disease, cigarette smoking, diabetes mellitus, pregnancy, hyperimmunoglobulinemia E syndrome, ataxia-telangiectasia, acquired immunodeficiency syndrome, and recurrent infections such as tuberculosis and aspergillosis (Boom et al., 1990; Drenth et al., 1994b; Hiemstra et al., 1989). Given these complexities, it is reasonable to take a combined approach in patients in whom there is a suitable index of suspicion for HIDS/mevalonate kinase deficiency, combining serum IgD measurement with genetic and/or biochemical analysis.

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

HIDS is inherited as an autosomal recessive trait. There are currently no data on the penetrance of individual HIDS genotypes, although population-genetic studies suggest that V377I homozygotes may not always manifest HIDS symptoms. In light of the likelihood of reduced penetrance, the generally favorable prognosis for HIDS, and the possibility of genetic discrimination, genetic testing is usually reserved for symptomatic individuals. Prenatal diagnosis of HIDS is possible but raises a number of ethical issues if undertaken with the possibility of terminating a pregnancy for what is usually a nonfatal disorder with a number of new treatment options.

TREATMENT AND PROGNOSIS

Despite the onset of symptoms in infancy, the average delay to diagnosis is 9.9 years, and a significant number of patients present with alternative diagnoses (van der Hilst et al., 2008a). The recurrent nature of the inflammatory episodes, especially for patients experiencing more than six attacks per year, may have an impact on social functioning and other quality-of-life measures (van der Hilst et al., 2008a). Thus, the therapeutic focus remains largely on prevention or suppression of inflammatory episodes.

Various approaches have been evaluated in an attempt to reduce inflammatory episodes and to modify the altered metabolic systems, but at the current time, there is no consensus on the treatment of HIDS. Most patients with HIDS do not respond to colchicine, although a few patients do show some improvement with daily treatment. Cyclosporine and intravenous immunoglobulin are not generally effective in HIDS. Corticosteroids at high doses at the onset of an attack may reduce its severity in a subset of patients (van der Hilst et al., 2008a).

More recently, therapeutic trials in HIDS have focused on the mevalonate pathway and on cytokine inhibition. The statin class of drugs inhibits HMG-CoA reductase, the enzyme immediately preceding mevalonate kinase in the mevalonate pathway. Although one member of this pharmacological family, lovastatin, caused exacerbations in the more severe mevalonic aciduria, simvastatin appears safe in HIDS, and preliminary data suggest a possible benefit (Attout et al., 2008; Simon et al., 2004b). Evaluation of the use of biological modulators in reducing the inflammation in HIDS is ongoing. A small trial of thalidomide, which, among other effects, inhibits TNF-α production, resulted in a nonsignificant decrease in acute phase reactants and no effect on the attack rate (Drenth et al., 2001b). In contrast, the more potent TNF inhibitor etanercept produced substantial improvement in three HIDS patients (Demirkaya et al., 2007; Takada et al., 2003), notably where treatment with corticosteroids and simvastatin had failed (Demirkaya et al., 2007). IL-1 inhibition may represent yet another possible therapeutic strategy. Use of anakinra in HIDS has led to the reduction or resolution of inflammatory attacks, decreased use of corticosteroids, and improvement in quality-of-life scores (Cailliez et al., 2006).

Furthermore, failure of efficacy of one biological agent does not eliminate the possible benefit from an alternate one (i.e. anakinra vs. etanercept; van der Hilst et al., 2008a).

Systemic amyloidosis remains a very rare complication of HIDS (D'Osualdo et al., 2004; Obici et al., 2004). Moreover, as noted above, symptoms usually ameliorate in adulthood, and the overwhelming majority of patients with HIDS have a normal life expectancy (Drenth et al., 1994b).

ANIMAL MODELS

Recently, two different murine models of HIDS have been reported. Hager et al. describe mice with loss of one allele of *Mvk* that demonstrate elevations of serum IgD, IgA, and TNF-α, consistent with reports of HIDS patients (Hager et al., 2007). Whereas complete knockout of the *Mvk* gene is embryonic lethal, the 50 percent reduction in mevalonate kinase activity is associated with accumulation of mevalonate in tissues, specifically the spleen, kidney, and heart. The associated splenomegaly and mild elevation in temperature suggest that this model is a reasonable phenocopy of human HIDS (Hager et al., 2007). Subsequent studies have demonstrated that these mice have altered expression of co-stimulatory molecules on T cells, B cells, and macrophages, as well as gender differences in the ability of splenocytes to proliferate in response to activation stimuli (Hager et al., 2012). These findings may lead to new information on the role of mevalonate kinase in inflammation.

In an alternate approach, postulating that the inflammatory mechanism in HIDS stems from the lack of isoprenoids rather than the accumulation of mevalonate, Marcuzzi et al. (2008) have evaluated mice with an MKD-like disorder induced by aminobisphosphonate, a mevalonate pathway inhibitor, and muramyldipeptide, a bacterial compound provided to mimic vaccine-induced episodes of inflammation. These mice demonstrate elevated SAA and peritoneal exudate cells, which resolved with administration of exogenous isoprenoids (Marcuzzi et al., 2008). Further studies using a similar approach in a murine monocyte cell line demonstrated that the combination of mevalonate pathway inhibition and bacterial stimulus led to programmed cell death and IL-1β secretion (Marcuzzi et al., 2011). More importantly, the cell death appears to be a combination of apoptosis and pyroptosis and could be rescued by treatment with anakinra (Marcuzzi et al., 2011). These findings may account for the resolution of symptoms observed in some HIDS patients following treatment with anakinra and provide in vitro support for the use of IL-1 receptor antagonists in patients with HIDS.

PERIODIC FEVER, APHTHOUS STOMATITIS, PHARYNGITIS, AND ADENITIS

In 1987, Marshall et al. described a series of 12 children with fevers that occurred with regular periodicity. In contrast to the hereditary recurrent fever disorders, these children displayed symptoms on a cyclical basis, with a period of 4.5 weeks on average, beginning prior to the age of 5 years and continuing through adolescence. Initially designated Marshall syndrome (Marshall et al., 1987) and subsequently FAPA, to include the associated symptoms of aphthous stomatitis, pharyngitis, and tender cervical adenopathy (Feder et al., 1989), the syndrome is now recognized as periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) to emphasize the characteristic periodicity of the febrile episodes.

Unlike the early descriptions of FMF and HIDS, which identified significant ethnic predilections, the original cohort comprised children from diverse ethnic ancestry, with mixtures of Northern European and Native American heritage. Additionally, none of the 12 children had a family history of recurrent fever disorders.

The molecular genetic etiology of PFAPA remains unknown. Children with PFAPA are noted to be not particularly susceptible to infection, clarifying that this unique syndrome is not an immunodeficiency but rather a dysregulation of the immune system, similar to the hereditary fever syndromes. Serum studies during febrile episodes in PFAPA patients have demonstrated elevated inflammatory cytokines, including IFN-γ and IL-18, which normalize during the asymptomatic period, consistent with the characteristic periodicity. Cytokines such as IL-1β, IL-6, and TNF-α remain elevated in the serum (Stojanov et al., 2006, 2011) compared to healthy controls, suggesting an underlying dysregulation of the innate immune system.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF PFAPA

PFAPA is classically recognized by its unique periodicity, in which patients have discrete episodes of high fevers, lasting 3 to 5 days per cycle, with a frequency of 10 to 11.5 febrile episodes per year (Thomas et al., 1999). Episodes begin prior to the age of 5 years, and most studies have demonstrated a slight male predominance (Thomas et al., 1999). As the name describes, in addition to fever, patients experience aphthous stomatitis, pharyngitis, and tender cervical adenopathy, although all may not be present in a given episode (Table 33.1). Nonspecific symptoms such as chills, headache, arthralgia, lethargy, and decreased appetite have also been described (Tasher et al., 2006; Thomas et al., 1999). Abdominal pain, nausea, and vomiting are more frequently observed with temperatures near 41°C but, unlike FMF, there is no serosal involvement (Marshall et al., 1987). The episodes are notable for the lack of upper respiratory symptoms. Rash and conjunctivitis (Kolokotronis et al., 2007) are rarely reported. The associated symptoms end abruptly with the fever, and patients are completely asymptomatic between episodes, with normal growth and development (Marshall et al., 1987). There is no identifiable trigger for the inflammatory episodes of PFAPA. Additionally, children with PFAPA are often noted to be less susceptible to infection (Marshall et al., 1987).

Although PFAPA is classically considered to be a syndrome of childhood, there are several reports of adults presenting with recurrent symptoms of fever, malaise, cervical

adenopathy, and exudative pharyngitis, similar to PFAPA (Colotto et al., 2011; Padeh et al., 2008). Compared to onset before the age of 5 years, adults with PFAPA less commonly experience aphthous stomatitis during their febrile episodes (Padeh et al., 2008). Of note, the delay in diagnosis tends to be longer in these patients (Padeh et al., 2008), possibly due to the increased age at onset. Additionally, one study reported that two thirds of adult patients with PFAPA experienced multiple, 2- to 6-month intervals that were symptom-free, thereby interrupting the characteristic periodicity of PFAPA syndrome (Padeh et al., 2008). It is possible that adult-onset PFAPA represents a unique subset on the spectrum of recurrent fever disorders, but evaluation of additional cases is necessary.

LABORATORY FINDINGS IN PFAPA

Similar to the other periodic fever syndromes, febrile episodes are marked by significant elevations in acute phase reactants, including ESR and CRP, which tend to be higher on days 2 to 4 of the fever cycle (Forsvoll et al., 2007). Normal to mildly elevated leukocyte counts with predominant neutrophilia have also been described (Thomas et al., 1999) but are not necessary for diagnosis (Forsvoll et al., 2007). These laboratory findings normalize completely between episodes, and there are no indications of subclinical inflammation. Quantitative immunoglobulins are within normal limits, although IgD may be elevated in a subset of PFAPA patients (Kovacs et al., 2010; Padeh et al., 1999). Similar to the hereditary fever disorders, autoantibodies are largely absent and complement studies are normal (Marshall et al., 1987). Cultures, such as pharyngeal swabs for *Streptococcus*, and serologies for *Toxoplasma*, *Mycoplasma,* Epstein-Barr virus, and cytomegalovirus are negative.

Recognition of the periodicity of fever episodes is of utmost importance in identifying PFAPA syndrome. Due to the frequency of the episodes, which occur once per month, and the early age at which symptoms develop, it is not uncommon for patients to be treated for viral illnesses, or empirically for streptococcal pharyngitis, otitis media, and sinusitis, with at least five courses of antibiotics (Forsvoll et al., 2007). The recurrent nature, high temperatures, and lack of identifiable source of the fevers may also prompt multiple subspecialty referrals. A child with PFAPA may have had evaluations by Infectious Diseases, Hematology, Oncology, Otolaryngology, Allergy/Immunology, and Rheumatology, as well as multiple visits to the primary pediatrician, over several years, before the diagnosis is reached.

MOLECULAR BASIS OF PFAPA

The etiology of PFAPA remains unknown. Whole-blood gene expression analysis and subsequent quantitative PCR has demonstrated upregulation of complement pathway genes, IL-1 and inflammasome-associated genes, and IFN-induced genes. In contrast, transcripts for receptors such as CXCR3, expressed on T lymphocytes, were downregulated compared to asymptomatic intervals (Stojanov et al., 2011). These findings indicate that at the level of gene expression, asymptomatic PFAPA patients are indistinguishable from healthy controls, and support the characteristic periodicity of this syndrome.

During febrile episodes, IFN-γ, IL-6, IL-18, G-CSF, IFN-inducible protein of 10 kDa (IP-10/CXCL10), monokine induced by IFN-γ (MIG/CXCL9), and macrophage inflammatory protein 1β (MIP-1β/CCL4) are significantly elevated in the serum (Brown et al., 2010; Stojanov et al., 2006, 2011). Further evaluation of cytokine profiles has demonstrated that patients with PFAPA have elevated IL-1β, IL-6, TNF-α, G-CSF, MIG/CXCL9, MIP-1β, and IL-12p70 between attacks compared to healthy controls (Stojanov et al., 2006, 2011). One of these genes, IP-10/CXCL10 (a chemoattractant for activated T cells and chemokines and chemokine receptors expressed by monocytes), was found to be a particularly good biomarker of disease.

At the cellular level, immunophenotyping has demonstrated that in addition to neutrophilia, numbers of monocytes, eosinophils, and activated $CD4^+$ lymphocytes are significantly altered during PFAPA flares, specifically a monocytosis with reduced numbers of eosinophils and $CD4^+HLA\text{-}DR^+$ and $CD4^+CD25^+$ T cells (Brown et al., 2010; Stojanov et al., 2011). There is no reported difference in activated CD8+ T cells during febrile episodes (Stojanov et al., 2011). These early studies demonstrate that inflammatory flares in PFAPA involve both innate and adaptive immune responses and confirm the hypothesis that immunodysregulation may be the basis of the PFAPA syndrome.

MUTATION ANALYSIS IN PFAPA

At the present time, a Mendelian inheritance pattern has not been clearly defined and no specific genetic mutation has been identified as the cause of PFAPA. The presence of the known periodic fever genes, specifically *MEFV*, *TNFRSFA1*, *CIAS1*, and the Crohn's disease-associated gene *CARD15/NOD2* in PFAPA patients has been examined. In one study, the frequency of the observed mutations in *MEFV* was not higher than the expected carrier frequency in their ethnic populations (Dagan et al., 2010). In another population, the presence of a single MEFV mutation, especially M694V, was associated with milder PFAPA episodes (Berkun et al., 2011). One patient did possess the R92Q mutation in *TNFRSFA1*; however, given the frequency of this mutation and the lack of other symptoms in the patient, it was considered more consistent with a genetic variant leading to mild or asymptomatic TRAPS (Dagan et al., 2010). Similarly, mutations in *CARD15/NOD2* have been shown to be similar in carrier frequency and less often associated with PFAPA than with Crohn's disease (Dagan et al., 2010; Maschio et al., 2006). These studies have been limited to evaluations of the predominant mutations rather than full gene sequencing. Whether the presence of these mutations, in the context of other genetic or environmental influences, leads to PFAPA remains to be determined.

STRATEGIES FOR THE DIAGNOSIS OF PFAPA

The diagnosis of PFAPA is made clinically, usually by exclusion of the hereditary periodic fever disorders previously described, lack of infections and lack of malignancy or a hematological process. The defining symptom of PFAPA is truly the characteristic periodicity of the fever cycles, which is not observed in the previously described fever syndromes. Criteria proposed by Thomas et al. (1999) have been traditionally used for diagnosis, specifically regularly recurring fevers with onset at less than 5 years of age and constitutional symptoms in the absence of upper respiratory infection with at least one of the following clinical signs: aphthous stomatitis, cervical adenitis, or pharyngitis. The patients must be completely asymptomatic during the interval between episodes and display normal growth and development. Exclusion of cyclic neutropenia is also recommended (Thomas et al., 1999).

The differential diagnosis of PFAPA includes viral infections, such as adenovirus and recurrent bacterial pharyngitis, both of which may present with fever, pharyngitis, and elevated CRP. However, even in cases of repeat infection, the lack of regular periodicity would distinguish between these illnesses and PFAPA. Behçet syndrome may also present with aphthous stomatitis, although these patients are less often febrile. Additionally, genital ulcers and iritis, which complete the classic Behçet's triad, would not typically be observed in PFAPA. Cyclic neutropenia may be the most similar to PFAPA in terms of regular febrile episodes. Cyclic neutropenia is a rare, autosomal dominant disorder that may be associated with mutations in the neutrophil elastase gene, *ELA-2*. It presents as episodes of fever, painful oral ulcers, and bacterial infections such as cellulitis, bronchitis, otitis, and sinusitis, secondary to severe neutropenia occurring every 3 weeks (Dale et al., 2011). Cyclic neutropenia may be complicated by chronic gingivitis, tooth loss, bacteremia, and septic shock, none of which is reported in PFAPA. Treatment is with subcutaneous granulocyte colony-stimulating factor.

A small case series has also associated PFAPA with Kawasaki syndrome in either the patient or a first-degree relative. Whether these specific cases point to variability in penetrance of a gene yet to be identified or the influence of environmental factors remains to be determined (Broderick et al., 2011). These cases also illustrate the difficulty in diagnosing children with recurrent fever disorders, given the necessity of episode recurrence for the clinical diagnosis.

PFAPA now appears to be more common than initially appreciated, with patients dispersed across subspecialties due to the chronic, persistent nature of the recurrent fever episodes. Evaluation of increasing numbers of patients with recurrent fevers has led to the recognition of a substantial number of patients with fevers of regular periodicity, similar to PFAPA, but without the associated symptoms of aphthous stomatitis, pharyngitis, or adenopathy. Parents of these children similarly report normal growth and development, negative microbial cultures, and a lack of response to antibiotics. We support the proposal by K. Barron to identify this subset of patients with the diagnosis of syndrome of undifferentiated recurrent fevers (SURF). It is too early to determine if these patients represent a milder form of PFAPA or if they will progressively display additional symptoms and merely represent an earlier stage of the PFAPA syndrome. Furthermore, the role of corticosteroids and/or tonsillectomy in these patients remains to be seen.

MODE OF INHERITANCE, CARRIER DETECTION, AND PRENATAL DIAGNOSIS

There are limited case reports describing PFAPA in siblings (Anton-Martin et al., 2011; Sampaio et al., 2009; Valenzuela et al., 2009), including one set of monozygotic twins and their mother (Adachi et al., 2011). Furthermore, it is not uncommon for parents of children with PFAPA to recall episodes of recurrent pharyngitis and fevers in their own childhoods (Cochard et al., 2010). Given the recent description of PFAPA only a few decades ago, no studies exist in which patients with PFAPA have been prospectively followed to determine whether *their* children would develop the syndrome. Moreover, for the vast majority of patients, a clear autosomal dominant pattern is not observed.

At the time of publication, no specific gene mutation has been identified for PFAPA, and carrier detection and prenatal diagnosis are not feasible. Despite the lack of specific genetic testing, parents should be reminded of the good prognosis for patients with PFAPA and the lack of known long-term morbidity or mortality.

TREATMENT AND PROGNOSIS

The prognosis for PFAPA is favorable. Despite the impact on quality of life due to monthly high fevers, there is no known mortality associated with PFAPA, and children have normal growth and development. Spontaneous resolution of inflammatory episodes occurred in over 40 percent of patients after a mean of 4.5 years in one cohort (Thomas et al., 1999). Longer courses of the syndrome are typically marked by increased intervals between fevers and less severe symptoms.

Medical treatment of febrile episodes in PFAPA is primarily supportive and targeted at reducing the high fevers. In terms of antipyretics, ibuprofen has demonstrated superiority over acetaminophen (Thomas et al., 1999). Antibiotic therapy has no utility in PFAPA episodes, and management should include avoiding unnecessary antibiotics. Numerous case reports have described alternative medical therapies aimed at remedying the syndrome, but the majority have been met with limited success. Colchicine therapy has been proposed as prophylactic therapy and in one series led to a significant increase in the asymptomatic interval in the majority of patients over a 2-year period (Tasher et al., 2008). A second small series demonstrated that twice-daily treatment with the H2-antagonist cimetidine may induce remission in a subset of patients (Feder, 1992; Thomas et al., 1999). Cessation of cimetidine therapy after 6 months, however, led to recurrence of symptoms in a few patients (Thomas et al., 1999). Prednisone has been used with considerable success in eliminating inflammatory episodes in PFAPA if given at the onset of fever. A single dose, typically 1 mg/kg, is effective in eliminating the fever within the first 24 hours of administration (Berlucchi et al., 2003). Associated symptoms, such as aphthous stomatitis, may take longer to resolve. This

remarkable response to corticosteroids may be considered an additional criterion aiding in the diagnosis of PFAPA. Unfortunately, the use of prednisone has also been associated with reducing the periodicity and shortening the interval between fever cycles in up to 50 percent of patients (Tasher et al., 2006; Thomas et al., 1999). The mechanism by which prednisone quells the fever episode, as well as by which corticosteroid therapy influences the cycle frequency, remains under evaluation. More recently, treatment of five PFAPA patients with subcutaneous anakinra, the recombinant IL-1R antagonist, on the second day of fever led to resolution of the fever and associated symptoms within hours of administration in three of the children (Stojanov et al., 2011). While this observation supports innate immune dysregulation as the mechanism behind the recurrent inflammatory episodes, further studies of the role of biological therapy is necessary.

Surgical intervention has been evaluated as an alternate therapy, and a potentially curative one, for PFAPA. In numerous case reports (Berlucchi et al., 2003; Licameli et al., 2008; Padeh et al., 1999; Peridis et al., 2010a, 2010b; Pignataro et al., 2009; Tasher et al., 2006; Thomas et al., 1999; Wong et al., 2008) and two small clinical trials (Garavello et al., 2009; Renko et al., 2007), tonsillectomy with or without adenoidectomy was shown to be effective in leading to clinical resolution. Adenoidectomy alone is not effective (Thomas et al., 1999), but it is unclear whether the combination of adenoidectomy with tonsillectomy is of additional benefit (Burton et al., 2010).

The mechanism behind the efficacy of tonsillectomy is unknown. Some have speculated that the palatine tonsils are responsible for immune dysregulation in PFAPA, and the removal of this active lymphatic tissue leads to clinical resolution. Follow-up in the current studies is variable, ranging from 3 to 41 months, and in some cohorts relapse occurred after a period of remission (Colotto et al., 2011; Tasher et al., 2006). Recurrence was proposed to be secondary to compensation by other oral lymphatic tissue (Colotto et al., 2011). In contrast, a smaller literature does not advocate tonsillectomy as a curative therapy for PFAPA. A single study of two patients reports diagnosis of PFAPA retrospectively following adenotonsillectomy (Parikh et al., 2003), and others have questioned the utility of surgical intervention for a syndrome that will likely resolve without long-term consequences (Leong et al., 2006). However, a recent meta-analysis comparing corticosteroid therapy and tonsillectomy did not demonstrate any statistically significant differences between the two approaches and confirmed the effectiveness of both in the treatment of PFAPA (Peridis et al., 2010b). Ultimately, the parents and clinical team must consider the risks and benefits of surgery against the alternative of recurrent episodes, which are likely to spontaneously resolve after a finite, but unknown, period of time.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The recurrent fever disorders represent a unique group of primary immune diseases characterized not by immunodeficiency and infection but by dysregulation of the innate immune system, resulting in inappropriate systemic and tissue inflammation. These conditions, now classified as autoinflammatory disorders, encompass several previously defined Mendelian fever disorders as well as other clinically related diseases with no known genetic basis. Recent advances in our understanding of the molecular basis of the hereditary fever disorders have helped to elucidate the complexity of the innate immune regulatory mechanisms, improved our diagnostic abilities, and led directly to effective targeted therapy of patients. In the future, identification of additional disease genes will help to elucidate the pathogenesis of the majority of recurrent fever patients who currently have no known defect.

For the practicing clinician, recognition of the shared and unique clinical characteristics of the recurrent fever syndromes is the initial step in diagnosis and management of patients with these disorders. Genetic testing can be used to confirm the diagnosis, define the prognosis, and aid in the selection of appropriate therapeutics from the many available targeted therapies to an individual patient.

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34.

INTRODUCTION TO INNATE IMMUNITY AND SYNDROMIC PRIMARY IMMUNODEFICIENCY DISORDERS

Jean-Laurent Casanova

Inborn errors of immunity, often referred to as primary immunodeficiencies (PIDs) in Europe and primary immunodeficiency diseases (PIDD) in the Americas, may be classified according to various criteria, including mode of inheritance (Mendelian vs. more complex traits), affected cell type (e.g., complement vs. B-cell deficiency), clinical phenotype (infectious vs. autoimmune), or age at onset (pediatric vs. adult-onset conditions). All these classifications are somewhat arbitrary and imperfect. Furthermore, they are not mutually exclusive, and this has paradoxically resulted in some confusion, even leading to the lack of an accurate, formal, and universal definition of PID/PIDD themselves. "PID" was historically used in a narrow, immunological, exclusive, and conservative sense (Fischer, 2007), whereas this term is now increasingly used in a broader, clinical, more inclusive, and liberal sense (Casanova and Abel, 2007). Despite this absence of a universal classification and definition of PIDs the field is rapidly evolving in at least two opposite directions, with the traditional search for new genetic etiologies for known PID phenotypes and the more recent discovery of new phenotypes resulting from inborn errors of immunity (Notarangelo and Casanova, 2009).

In this context, perhaps the most consensual but least rigorous classification, combining some but not all of these criteria in a somewhat inconsistent manner, is that adopted by the International Union of Immunological Societies (IUIS) expert committee for PIDs. This classification assigns PIDs to eight broad categories: "combined T- and B-cell deficiencies," syndrome PIDs (often T-cell defects), "antibody deficiencies," "diseases of immune dysregulation," "defects of phagocytes," "defects of innate immunity," "autoinflammatory disorders," and "complement deficiencies"(Al-Herz et al., 2011). The classification of PIDs adopted in this textbook was inspired by this IUIS classification. This brief introduction precedes two sections dealing with "syndromic PIDs" and "PIDs of innate immunity," corresponding approximately to Tables II and VI of the IUIS classification. "Syndromic PIDs" is the term used to describe PIDs with clinical features extending well beyond the immunological boundaries of PIDs. Several important "syndromic PIDs" are described in other sections (e.g., ataxia-telangiectasia), and the immunological features of these "syndromic PIDs" differ considerably between conditions (ranging from T-cell to phagocyte defects). It is therefore very difficult to define this group of PIDs, which encompasses diseases as diverse as cartilage-hair hypoplasia (CHH), hyper-IgE syndromes (HIES), hepatic veno-occlusive disease with immune deficiency (VODI), WHIM syndrome, pulmonary alveolar proteinosis, epidermodysplasia verruciformis, and Wiskott-Aldrich syndrome (WAS), but not anhidrotic ectodermal dysplasia with immune deficiency, Schimke disease, Fanconi anemia, or ataxia-telangiectasia.

The "PIDs of innate immunity" selected in this section are even more difficult to define, especially given that the only two chapters assigned to this section concern "disorders of the IL-12-IFN-γ circuit" and "defects of the NF-κB pathway." In the immunological lexicon, adaptive immunity refers to the antigen-specific, lymphoid immunity mediated by T and B cells. By contrast, innate immunity refers to NK lymphocytes and to myeloid cells, comprising polymorphonuclear phagocytes, including neutrophil, eosinophil, and basophil granulocytes, and mononuclear phagocytes, including various types of monocytes, macrophages, and dendritic cells. Continuing a longstanding immunological tradition, this dichotomy omits key players in host defense, such as the nonhematopoietic cells present in mucocutaneous barriers and internal organs. This arm of nonhematopoietic immunity is sometimes referred to as "intrinsic immunity," although this term is ambiguous to some extent, as antiviral intrinsic immunity mechanisms also

occur in leukocytes themselves (Bieniasz, 2004). In any event, there is no obvious reason why "disorders of phagocytes" or "disorders of complement" should be distinguished from those of "innate immunity," particularly when the only two PIDs from the innate immunity group dealt with here have a major effect on adaptive immunity. This effect is probably even stronger than those on innate and intrinsic immunity, as IL-12 is a key activator of T cells and IFN-γ is a key T-cell cytokine, with NF-κB being used as a transcription factor by multiple T- and B-cell receptors.

These difficulties are inevitable and reflect the fact that this classification of PID is based largely on a cellular classification of immunity, corresponding to nonhematopoietic (intrinsic), myeloid (innate), and lymphoid (adaptive) cells. Adaptive immune responses are clonal, antigen-specific, diverse, and endowed with memory. Innate immunity is considered to differ from adaptive immunity on each of these points. Indeed, this historical view of immunology revolves around the antigen, which defines antigen-specific (adaptive) immune responses as the quintessential basis of immunity, with little consideration of the contribution of nonspecific (innate) immune responses, and the banishment of other (intrinsic) layers of host defense to obscurity. However, these three arms of the immune system are closely connected and a defect in any one of these arms may affect the other two. For example, defects of antigen-specific cells may affect pathogen-digesting effector cells, and defects of antigen-presenting cells may affect antigen-specific lymphocyte responses. Would this justify a classification based on the distinction between intrinsic, innate, and adaptive inborn errors of immunity? In fact, the terms "intrinsic," "innate," and "adaptive" are appropriate when we are referring to the cells involved in host defense, but they are not entirely appropriate for use with genes, and thus for inborn errors of immunity and PIDs.

Indeed, only a very small number of genes can be viewed as truly and purely "adaptive": those encoding RAG, TCR, BCR, HLA-I, and HLA-II molecules and perhaps a few associated molecules. These "adaptive" molecules define about 20 to 30 genes, 50 at most if we also include genes that are almost exclusively functional in T and B cells. Does this imply that the remaining 20,950 human genes (from a total of about 21,000 genes, without even considering the noncoding DNA segments) are innate or intrinsic? Most of these genes are expressed in both innate and adaptive cells, not to mention nonhematopoietic cells. As a result, only a handful of known PIDs, such as HLA-I, HLA-II, CD3, and RAG defects, are purely adaptive. Most other PIDs affect both innate and adaptive immunity or, more rarely, innate immunity alone. Some recently described PIDs even affect intrinsic immunity, alone or in combination with hemopoietic immunity. Conversely, very few PIDs are purely innate or intrinsic, because the morbid genes are also expressed in B and T cells. As a result, the chapters covered in this section of the textbook cover disorders typically affecting all three types of immunity (e.g., NF-κB disorders, errors of IL12-IFN-γ, CHH, HIES, and WAS). Complicating matters even further, these disorders have also been somewhat arbitrarily selected, with some being exceedingly rare (VODI) and others more common (HIES); some being genetically homogeneous (CHH) and others extremely heterogeneous (IL-12-IFN-γ deficiencies); and some being defined in terms of a remotely shared cellular phenotype (NF-κB) and others in terms of their clinical phenotype (WAS).

So, readers should probably avoid trying to decipher the rationale underlying the classification of PIDs throughout this book, including their assignment to two groups. The truth is that there is no clear-cut rationale for PID classification, and a random selection of chapters and sections, and a random distribution of chapters within sections, would undoubtedly have generated as coherent a table of contents as that presented here. For example, the terms "syndromic PIDs" and "PIDs of innate immunity" cannot adequately convey the content of these two sections. Is this incoherence a problem? We don't think so. Indeed, it elegantly illustrates the current lack of a consistent paradigm in the field of PIDs, and the current drifting of this field, with no rigorous definition and no appropriate classification. We know from the history of science that such crises generally precede a profound shift of paradigm (Kuhn, 1962). The extraordinarily rapid expansion of knowledge about PIDs over the past 20 years has thrown up increasing numbers of exceptions to the paradigm first defined in the 1950s (reviewed by Casanova and Abel, 2007). The discovery of new genotypes and phenotypes has called previous definitions and classifications of PIDs into question. This crisis is undoubtedly the harbinger of a revolution. There is currently no better definition and classification available than that presented here—no new paradigm to make sense of these data. In this context, the next two sections will provide the reader with an overview of the current crisis in the field of PIDs, perhaps more so than the other sections, which have been less disturbed by recent findings.

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35.

INHERITED DISORDERS OF THE INTERLEUKIN-12–INTERLEUKIN-23/ INTERFERON-γ CIRCUIT

Steven M. Holland and Jean-Laurent Casanova

The recognition of severe disseminated infections with weakly virulent mycobacteria in individuals without recognized predisposing immunodeficiency as well as high rates of affected siblings and parental consanguinity suggested the existence of a novel set of primary immunodeficiency syndromes, subsequently subsumed under the name *Mendelian susceptibility to mycobacterial disease* (MSMD, MIM 209950). Mycobacteria involved include many species of environmental nontuberculous mycobacteria (NTM) and several *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccine substrains. Patients with MSMD are also prone to develop tuberculosis. Molecular investigation of these families has so far identified mutations in seven genes in the interleukin (IL)-12-dependent interferon-gamma (IFN-γ) circuit, one of the genes in the respiratory burst pathway, and one gene in the early hematopoietic pathway, highlighting the importance of these pathways in human immunity to mycobacteria and select other intramacrophagic pathogens. However, there remain patients for whom genetic etiologies are likely in whom mutations have not yet been identified, indicating that other morbid genes are yet to be found.

Sporadic cases of disseminated NTM infection in the absence of recognized immunodeficiency have been well described over many decades (Buhler and Pollack, 1953; van der Hoeven et al., 1958). Familial-disseminated NTM infection was first reported in 1964—three members of the same Danish family had fatal disseminated *Mycobacterium avium* complex (MAC) (Engbaek, 1964). Uchiyama et al. (1981) identified two siblings with *M. avium* infection. Holland et al. reported three male members of one family with disseminated NTM infection (Frucht and Holland, 1996; Frucht et al., 1999; Holland et al., 1994).

Idiopathic disseminated BCG infection following vaccination was first reported in 1951 (Mimouni, 1951). A sporadic case born to consanguineous parents was described in 1973 (Ulgenalp et al., 1973), and families with more than one affected member were first reported at around the same time (Fedak, 1974; Sicevic, 1972). Patients with inherited susceptibility to mycobacterial infections may also be at increased risk of *Salmonella* infection, as first highlighted by Heyne (1976), who described a brother and sister from Germany with generalized infection after neonatal BCG vaccination. The boy later developed *Salmonella* enteritis and osteomyelitis. In Prague, a 3-year-old boy who had been vaccinated with BCG at 3 days of age developed disseminated *Salmonella* and BCG infection, resulting in his death 3 years later (Doleckova et al., 1977). A first cousin of this child also had disseminated BCG infection (Dvoracek et al., 1959).

The first family in which the molecular basis of susceptibility to NTM was elucidated was described in 1995 (Levin et al., 1995). Four children from the same village in Malta all developed disseminated NTM infection. Two were brothers related to a third child as fourth cousins, while the fourth child was not knowingly related to the others. Each child was infected with a different mycobacterial species (*M. chelonae*, *M. fortuitum*, and two different strains of MAC). Extensive immunological investigation failed to identify any known defect predisposing to such infections. However, the patients had defective monocyte responses to IFN-γ when added to endotoxin (Levin et al., 1995) and defective antigen presentation (D'Souza et al., 1996). The high degree of consanguinity within the Maltese family suggested that the children were homozygous for a rare recessive mutation inherited from a common ancestor. A whole-genome search in three of the affected children mapped a homozygous region of chromosome 6q containing the gene encoding the IFN-γ receptor ligand binding chain (IFN-γR1) of the IFN-γ receptor complex (Newport et al., 1996), and sequence analysis identified a mutation in the extracellular domain of *IFNGR1* resulting in complete absence of IFN-γR1 expression on the cell surface.

Casanova et al. (1995) surveyed 108 cases of disseminated BCG infection following vaccination reported since 1951, finding that fully 50 percent were not accounted for by previously recognized molecular defects. A retrospective study of all cases of disseminated BCG infection following vaccination in France between 1974 and 1994 confirmed that of 32 children identified, 16 had no recognized predisposing immunodeficiency (Casanova et al., 1996). Further, among 60 children worldwide with idiopathic disseminated BCG infection for whom information was available, four pairs of siblings and one pair of first cousins were identified, and parental consanguinity was noted in 24 families. Clinical and histopathological features in a Tunisian child with disseminated BCG infection, born to consanguineous parents, were remarkably similar to those of the Maltese children with NTM infections. A series of candidate genes involved in antimycobacterial immunity in the mouse model of BCG infection were tested by homozygosity mapping. The segregation of markers within *IFNGR1* suggested linkage, and a frameshift mutation resulting in the absence of IFN-γR1 was identified (Jouanguy et al., 1996).

Although mutations in *IFNGR1* were subsequently identified in other cases of MSMD, there were a number of patients in whom mutations within *IFNGR1* were not detected. Investigation of other candidate genes involved in IFN-γ–mediated immunity led to the identification of mutations in seven genes (Table 35.1) involved in IL-12–dependent, IFN-γ–mediated immunity, one involved in NADPH oxidase activity (*CYBB*), and one involved in early hematopoiesis (*GATA2*) (Al-Muhsen and Casanova, 2008; Bigley et al., 2011; Bustamante et al., 2011a; Casanova and Abel, 2002, 2004; Dickinson et al., 2011; Dorman and Holland, 2000; Filipe-Santos et al., 2006a; Fortin et al., 2007; Hsu et al., 2011; Rosenzweig and Holland, 2005; Vinh et al., 2010).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

The central feature of MSMD is infection with NTM or BCG, weakly pathogenic mycobacteria to which exposure is common and disease rare. There is a clinical spectrum of MSMD that closely follows the extent of residual function of the affected gene: at one end of the spectrum are complete loss-of-function mutations in *IFNGR1* or *IFNGR2*, which result in no functional protein at the cell surface (complete receptor deficiency). These complete defects lead to the development of disseminated infection in early childhood and usually progressively fatal disease (Dorman and Holland, 1998; Dorman et al., 2004; Jouanguy et al., 1996; Newport et al., 1996). At the other end of the spectrum, *IFNGR1* and *IFNGR2* mutations that permit the expression of abnormal but somewhat functional protein cause partial receptor deficiency and are associated with milder phenotypes that retain some response to IFN-γ (Doffinger et al., 2000; Dorman et al., 2004; Jouanguy et al., 1997, 1999b). There is a correlation between the cellular phenotype (response to IFNγ) and the clinical phenotype (severity of mycobacterial disease) (Dupuis et al. 2000). Recessive mutations in the genes encoding the IL-12p40 subunit (*IL12B*) or the IL-12 receptor β1 subunit (*IL12RB1*) resulting in complete deficiency of either protein generally result in a less severe phenotype and good response to antibiotics and IFN-γ treatment. Likewise, dominant negative mutations in signal transducer and activator of transcription 1 (*STAT1*) or IRF8 (Hambleton et al., 2011) cause a partial cellular defect and relatively mild clinical phenotypes (Dupuis et al., 2000). For mutations with some residual function, screening of asymptomatic family members has identified individuals who carry mutations but have not developed infection with either mycobacteria or *Salmonella*, indicating that the clinical penetrance is incomplete, unlike in families with complete defects in *IFNGR1*, *IFNGR2*, or *STAT1* (Altare et al., 2001; Caragol et al., 2003; Dupuis et al., 2001; Jouanguy et al., 1999b; Picard et al., 2002).

One of the most striking features of MSMD is the relatively narrow susceptibility to poorly pathogenic mycobacterial species, including slow-growing species (e.g., *M. kansasii*, *M. avium*, *M. scrofulaceum*, and *M. szulgai*) and rapid-growing species (e.g., *M. smegmatis*, *M. abscessus*, *M. chelonei*, *M. fortuitum*, *M. peregrinum*) (Koscielniak et al., 2003; Marazzi et al., 2010; Pierre-Audigier et al., 1997). The more virulent *M. tuberculosis* has been implicated in or isolated from individuals

Table 35.1 **GENES INVOLVED IN DEFECTIVE MACROPHAGE ACTIVATION**

GENE	GENE PRODUCT	CHROMOSOMAL LOCATION	MIM NO. (PHENOTYPE MIM NO.)
IFNGR1	Interferon-γ receptor ligand binding chain	6q23.3	107470 (209950)
IFNGR2	Interferon-γ receptor signal transducing chain	21q22.1–2.2	147569 (209950)
IL12RB1	Interleukin-12 receptor β-1 subunit	19p13.11	601604 (209950)
IL12B	Interleukin-12p40 subunit	5q33.3	161561
STAT1	Signal transducer and activator of transcription 1	2q32.2	600555 (209950)
CYBB	Cytochrome b; NADPH oxidase 2 (NOX2), $gp91^{phox}$	Xp11.4	300645
IKBKG	NFκB essential modulator (NEMO), IKK-G	Xq28	300248 (300636)
IRF8	Interferon regulatory factor 8	16q24.1	601565
GATA2	GATA binding protein 2	3q21.3	137295 (614172)

with IFN-γR1, IL-12p40, and IL-12Rβ1 deficiencies and patients with unique mutations in gp91^{phox} (Altare et al., 2001; Boisson-Dupuis et al., 2011; Bustamente et al., 2011a; Caragol et al., 2003; Jouanguy et al., 1997; Özbek et al., 2005; Picard et al., 2002). Haploinsufficient mutations in the *GATA2* gene predispose to infections with both rapid- and slow-growing mycobacteria (Bigley et al., 2011; Vinh et al., 2010). The mycobacterial species isolated seem to track with the underlying genetic defect to some extent: rapid-growing mycobacteria are mostly recovered from children with complete IFN-γR1 or IFN-γR2 deficiencies, whereas they are quite uncommon in children or adults with partial defects, suggesting that even a small amount of residual IFN-γR activity is enough to keep rapid-growing mycobacteria at bay (Table 35.2).

Despite the predominance of NTM in MSMD, they are not the only opportunistic infections encountered. *Salmonella* infections ranging from protracted gastroenteritis to septicemia and disseminated infection occurred in about a quarter of cases, more commonly in association with IL-12p40 and IL-12Rβ1 deficiency than IFN-γR defects (MacLennan et al., 2004). Other pathogens isolated from MSMD cases include *Listeria monocytogenes* (Roesler et al., 1999), *Histoplasma capsulatum* (Jouanguy et al., 1999b; Zerbe and Holland, 2005), *Coccidioides immitis* (Vinh et al. 2009, 2011), *Nocardia asteroides* complex (Picard et al., 2002), *Nocardia* spp. (Luangwedchakarn et al., 2009), *Klebsiella pneumoniae* (Pedraza et al., 2010), *Kingella kingae* (Staretz-Haham et al., 2003), and *Leishmania* spp. (Sanal et al., 2007). Filamentous fungi and bacterial pathogens such as staphylococci have not caused infection in MSMD, despite the presence of indwelling intravenous catheters in many patients. However, a substantial fraction of patients with IL-12p40 and IL-12Rβ1 deficiency (deBeaucoudry et al., 2008, 2010), but not other forms of MSMD, have some forms of chronic mucocutaneous candidiasis, probably related to impairment of their IL-23/IL-17 circuit (Puel et al. 2010, 2011).

Increased susceptibility to viral infections, particularly herpes viruses, has been noted in some patients with IFN-γR1 deficiency (Cunningham et al., 2000; Dorman et al., 1999; Novelli and Casanova, 2004; Roesler et al., 2011; Uzel et al., 2000). One 11-year-child with complete IFN-γR1 deficiency who had developed mycobacterial disease at the age of 5 months was diagnosed with human herpesvirus-8 (HHV-8)-associated Kaposi sarcoma (Camcioglu et al., 2004). However, most other MSMD patients have had classical childhood viral infections without problems. Partial functional mutations in *STAT1* have not resulted in increased susceptibility to viral infection despite the role of *STAT1* in both IFN-γ– and IFN-α–mediated immunity (see the section "Molecular Basis of Disease," below).

Patients with MSMD due to complete IFN-γR1 deficiency often present in childhood with a characteristic syndrome of chronic fever, weight loss, lymphadenopathy, and hepatosplenomegaly due to disseminated infection. The clinical presentation varies according to the gene defect and its severity. For example, dominant partial IFN-γR1 deficiency is almost always associated with NTM osteomyelitis (Dorman et al., 2004; Jouanguy et al., 1999b; Sasaki et al., 2002; Villela et al., 2001; Vinh et al., 2009; Zerbe and Holland, 2005), whereas lymphadenopathy is a very common feature of IL-12p40 or IL-12Rβ1 deficiency (Aksu et al., 2001; Altare et al., 1998a, 1998c, 2001; de Jong et al., 1998; Picard et al., 2002; Sakai et al., 2001). The distinct clinical features of each genetic defect will be more fully described as more patients with MSMD are identified. The age of onset varies according to the gene involved, the type of mutation, and whether the affected individual received BCG vaccination at birth or acquired NTM infection via natural routes (Dorman et al., 2004). A correlation between clinical phenotype and histopathology has been observed (Emile et al., 1997). Two distinct histological types have been documented that appear to be associated with distinct clinical phenotypes (Color Plate 35.I).

Approximately half the patients with disseminated BCG infections had tuberculoid (type I) granulomata with well-defined epithelioid giant cells surrounded by lymphocytes and fibrosis containing only occasional acid-fast bacilli. The remaining patients had lepromatous-like (type II) lesions with poorly formed granulomata containing large numbers of acid-fast bacilli. Patients with type I granulomata had a good prognosis, but virtually all the children with poor granuloma formation (type II) died. NTM granulomata tend to be poorly formed irrespective of the clinical outcome and underlying genetic defect.

LABORATORY FINDINGS

Chronic infection leads to normochromic, normocytic anemia and raised inflammatory markers. Immune function in patients with MSMD has been extensively investigated in an attempt to identify a known immunodeficiency and is in general remarkably normal. $CD4^+$ T cells are often normal but may be low, probably due to chronic infection. Levels of serum immunoglobulins, including IgG subclasses, are normal or elevated, and antigen-specific antibody titers are normal. T-cell proliferation in vitro to various mitogens and recall antigens is also normal. Polymorphonuclear neutrophils are normal in terms of morphology, CD18 expression, chemotaxis, and respiratory burst, even in the case of the CYBB mutations associated with MSMD. Delayed-type hypersensitivity (DTH) testing in vivo and blastogenesis in vitro to purified protein derivative (PPD) are normal, even in patients with complete IFN-γR1 and IL-12Rβ1 deficiency, indicating that IL-12 and IFN-γ are not required for DTH or blastogenesis to mycobacterial antigens.

MOLECULAR BASIS OF THE DISEASE

Mutations in seven genes of the IL-12/IFN-γ axis, and one each in the NADPH oxidase and in the primitive hematopoietic pathway causing increased susceptibility to mycobacteria, have been identified to date (summarized in Fig. 35.1). In humans, these pathways are central to the immune response to intramacrophagic pathogens such as mycobacteria, *Salmonella*, and certain dimorphic yeasts.

Table 35.2 CLINICAL FEATURES OF INHERITED DISORDERS OF INTERLEUKIN-12–INTERFERON-γ/IL-12 CIRCUIT: PATHOGENS ISOLATED AND ORGANS INVOLVED

DISORDER	INFECTION	RES	BONE	CNS	GIS	RS	SKIN	REFERENCES
cIFN-γR1	MAC	+	+	+		+		Newport et al., 1996;
	M. fortuitum	+	+					Jouanguy et al., 1996, 2000
	M. chelonae	+	+					Pierre-Audigier et al., 1997
	BCG	+	+	+			+	Altare et al., 1998b
	M. smegmatis	+		+				Holland et al., 1998
	M. kansasii	+	+			+	+	Vesterhus et al., 1998
	M. szulgai	+	+					Roesler et al., 1999
	Salmonella	+			+			Cunningham et al., 2000
	L. monocytogenes	+		+				Allende et al., 2001
								Rosenzweig et al., 2002; Koscielnak et al., 2003
cIFN-γR2	*M. fortuitum*	+				+		Dorman and Holland, 1998
	MAC	+				+		
AR pIFN-γR1	BCG	+					+	Jouanguy et al., 1997
	Salmonella	+						
AR pIFN-γR2	BCG	+					+	Doffinger et al., 2000
	M. abcessus	+					+	
AD pIFN-γR1	*M. tuberculosis*	+				+		Jouanguy et al., 1999b
	MAC		+			+	+	Villela et al., 2001
	BCG	+	+			+	+	
	M. kansasii	+						
	Salmonella		+					
	H. capsulatum	+	+					Zerbe and Holland, 2005
	C. immitis	+	+					Vinh et al., 2009
cIL-12p40	BCG	+					+	Altare et al., 1998c; Picard et al., 2002
	M. tuberculosis	+						
	Salmonella spp.	+			+			
	Nocardia spp.					+		
cIL-12Rβ1	BCG	+						Altare et al., 1998a, 2001
	MAC	+				+		de Jong et al., 1998
	M. fortuitum-chelonae	+			+	+		Asku et al., 2001; Sakai et al., 2001
	M. tuberculosis	+			+			
	Salmonella	+			+			
	C. immitis	+						Vinh et al., 2011
STAT1	BCG	+						Dupuis et al., 2001
	MAC	+						
	Lethal viral infection	+/-						Dupuis et al., 2003
gp91^{phox}	BCG	+/-						Bustamante et al., 2011
NEMO	BCG	+/-	+/-					Hanson et al., 2008
	NTM	+/-	+/-			+/-		
	CMV	+/-			+/-			
	S. pneumoniae	+/-		+/-				
	P. aeruginosa	+/-						
IRF8	BCG	+/-						Hambleton et al., 2011
GATA2	NTM	+/-	+/-			+/-	+/-	Vinh et al., 2010; Hsu et al., 2011
	C. neoformans	+/-		+/-				

(continued)

Table 35.2 (CONTINUED)

DISORDER	INFECTION	RES	BONE	CNS	GIS	RS	SKIN	REFERENCES
	A. fumigatus					+/-	+/-	
	H. capsulatum	+/-				+/-		
	Human papillomavirus						+/-	

gp91phox (see the formatting in text in Table 35.2), catalytic subunit of NADPHoxydase; NEMO, NF-kB essential modulator, transcription factor; IRF8, interferone regulatory factor 8, transcription factor; GATA2, gata binding protein 2.

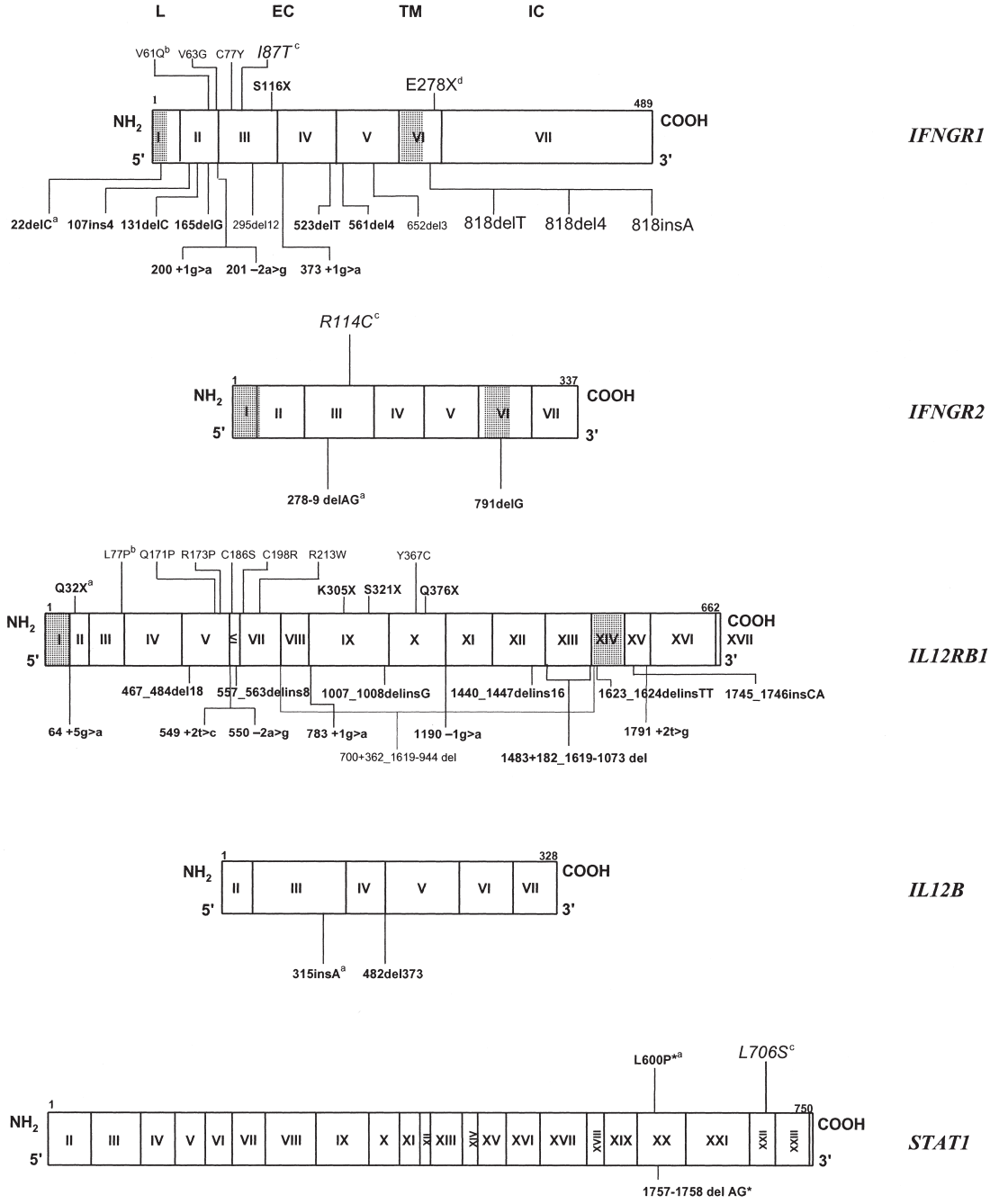

Figure 35.1 Mutations identified to date in five of the nine known MSMD genes. The gene-coding regions are indicated with vertical bars separating the exons, designated by Roman numerals. [a]Nonsense, splice-site mutations, and frameshift insertions and deletions (recessive) causing complete deficiency with no detectable protein expression at the cell surface (small font in bold); [b]missense mutations and in-frame deletions (recessive) causing complete deficiency with detectable surface protein expression (small font); [c,d]mutations that are recessive (large font, italic)[c] or dominant (large font),[d] causing partial deficiency; *indicates two patients with homozygous STAT1 mutation who were susceptible to not only mycobacterial but also severe or eventually lethal viral infections.

FUNCTIONAL ASPECTS OF THE PROTEINS

The receptor for IFN-γ consists of two subunits: IFN-γR1, the ligand-binding chain (previously known as the α chain), and IFN-γR2, the signal-transducing chain (previously known as the β chain or accessory factor 1) (Bach et al., 1997). When the ligand-binding chains interact with IFN-γ homodimers they dimerize and become associated with two signal transduction chains. This leads to the activation of specific members of two protein families—the Janus kinases (JAK) and the signal transducers and activators of transcription (STAT). JAK1 and JAK2 phosphorylate key tyrosine residues on the intracellular domains of the ligand-binding chains. This leads to the recruitment, phosphorylation, and dimerization of STAT1, which translocates to the nucleus as a phosphorylated homodimer (also known as gamma activating factor [GAF]) to activate a wide range of IFN-γ-responsive genes. Subsequently, the phosphorylated STAT1 dimers are dephosphorylated and released from the DNA. After signaling, the receptor complex is internalized and dissociates. The IFN-γR1 chain is recycled to the cell surface, while the IFN-γR2 is more selectively expressed. IFN-γR1 is expressed constitutively at moderate levels on the surface of all nucleated cells. IFN-γR2 is also constitutively expressed at low levels but expression is regulated by external stimuli, including IFN-γ itself (Bach et al., 1995).

Interleukin-12 is a heterodimeric cytokine composed of two subunits, p40 and p35, which together form the biologically active p70 molecule. The IL-12p40 subunit is shared by IL-23 (Fieschi and Casanova, 2003). IL-12 and IL-23 are produced by activated antigen-presenting cells such as dendritic cells and macrophages in response to a number of microorganisms and microbial products, including lipopolysaccharide, lipoarabinomannan, and bacterial superantigens (Trinchieri, 1998). It can also be secreted upon stimulation by T cells in a CD154(CD40L)-CD40–dependent and IFN-γ–dependent manner. The stimulation of whole blood by live BCG was shown to trigger the IL-12/IFN-γ axis by an IRAK-4– and NEMO-dependent, noncognate interaction among monocytes and natural killer (NK) cells and T lymphocytes (Feinberg et al., 2004). It promotes cell-mediated immunity to intracellular pathogens by inducing the production of IFN-γ by both T and NK cells. The IL-12 receptor complex, expressed on activated T and NK cells, consists of two subunits known as the β1 and β2 subunits. Binding of IL-12 to the heterodimeric IL-12 receptor complex induces the phosphorylation of tyrosine kinase 2 (Tyk2) and JAK2 and subsequent activation of STAT4, which dimerizes and translocates to the nucleus to activate IL-12–inducible genes. The IL-12Rβ1 subunit is also part of the IL-23 receptor. Figure 35.2 shows the cytokine interactions between the macrophage and T or NK cell in the context of mycobacterial infection, illustrating the interaction between IL-12 and IFN-γ. IRF8 is an IFN-γ inducible transcription factor required for the induction of various target genes, including IL-12. Gp91^{phox} is the critical transmembrane X-linked component of the phagocyte NADPH oxidase. GATA2 is a critical transcription factor that drives early hematopoiesis as well as mature macrophage function.

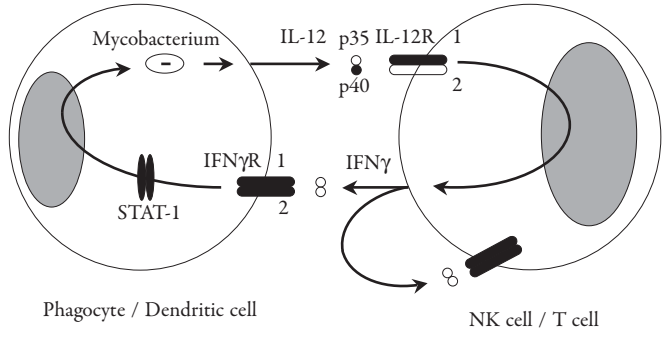

Figure 35.2 Cytokine interactions between the macrophage or dendritic cell and T or NK cell in the context of mycobacterial infection, illustrating the interaction between IL-12 and IFN-γ. Upon infection, primary host response cells such as macrophages release a range of cytokines, including IL-12, which stimulate T and NK cells to secrete IFN-γ, activate m-acrophages to kill intracellular pathogens, and enhance the differentiation of IFN-γ–producing T helper cells.

IFNGR1

Mutations in this gene were the first to be identified as the cause of MSMD (Jouanguy et al., 1996; Newport et al., 1996). Subsequent investigation of patients with increased susceptibility to poorly pathogenic mycobacteria has led to the identification of numerous null-recessive mutations in this gene (Fig. 35.1) (Allende et al., 2001; Altare et al., 1998b; Cunningham et al., 2000; Dorman et al., 2004; Holland et al., 1998; Jouanguy et al., 2000; Koscielniak et al., 2003; Noordzij et al., 2007; Pierre-Audigier et al., 1997; Roesler et al., 1999; Rosenzweig et al., 2002; Vesterhus et al., 1998). In one patient complete IFN-γR1 deficiency was caused by paternal uniparental disomy of chromosome 6 (Prando et al., 2010). The identification of families in which mycobacterial infections occurred in more than one generation suggested that dominant mutations might also exist (Jouanguy et al., 1999b; Han et al., 2004). Investigation of 18 individuals from 12 kindreds led to the identification of a small deletion hot spot within *IFNGR1*. A 4 base pair (bp) deletion at nucleotide position 818 (818del4) was identified in 11 of the unrelated kindreds, and the 12th family had a single nucleotide deletion (T) in this position (818delT) (Glosli et al., 2008). The 818del4 (818delT) mutation leads to a premature stop codon at position 827–829 within the intracellular domain of the receptor. The receptor is expressed on the cell surface, but the mutant receptor lacks the three motifs required for intracellular signaling (the JAK1 and STAT1 binding sites, and the tyrosine phosphorylation site). It also lacks a recycling motif, so the truncated receptor accumulates on the cell surface and interferes with signaling by the normal receptor encoded by the normal copy of *IFNGR1*. Thus, the mutant allele has a dominant negative effect (in comparison to the recessive form of IFN-γR1 deficiency, in which parents are healthy carriers of the mutations). Subsequently, other *IFNGR1* mutations resulting in a dominantly inherited phenotype were identified (Dorman et al., 1999; Sasaki et al., 2002; Villella et al., 2001). A second small deletion hot spot was recently identified in *IFNGR1*, in this case with a recessive phenotype (Hoshina et al., 2011; Rosenzweig et al., 2002).

In summary, a range of mutations, including frameshift, insertion, deletion, nonsense, missense, and splice mutations, have been identified in *IFNGR1* (Fig. 35.1). All recessive mutations identified to date occur in the part of the gene encoding the extracellular domain of the receptor chain, the majority of which result in complete lack of receptor expression. Two of the recessive mutations allow expression of a poorly functioning protein, leading to partial deficiency (Jouanguy et al., 1997; Kong et al., 2010a ; Sologuren et al., 2011). Partial recessive receptor deficiency is more common than previously thought. Partial defects may also result from dominant mutations that cause the receptor to be deprived of its intracytoplasmic segment and therefore neither able to signal properly nor leave the plasma membrane, leading to overaccumulation of a mutant inhibitory protein.

IFNGR2

Complete deficiency of IFN-γR2 was found in a child with disseminated *M. fortuitum* and MAC infections in whom cell-surface expression of IFN-γR1 and *IFNGR1* sequence were normal (Dorman and Holland, 1998). Sequence analysis of *IFNGR2* led to the identification of a homozygous 2 bp deletion (277–278delAG), which in turn led to a premature stop codon (Fig. 35.1). The truncated protein lacked both the transmembrane and intracellular (signaling) domains and was not expressed at the cell surface. Both parents, though unrelated, carried this mutation without cellular phenotype. Another recessive complete 2-base deletion (949delTG) with severe recurrent mycobacterial disease and adolescence-onset diffuse squamous cell cancer has also been reported (Toyoda et al., 2010), confirming the importance of the IFNγ/IL-12 pathway in cancer surveillance. A case of partial IFN-γR2 deficiency has also been described (Doffinger et al., 2000) due to an amino acid substitution at position 114 (arginine → cysteine) within the extracellular domain (Fig. 35.1). The mutant IFN-γR2 is expressed normally on the cell surface, but presumably the affinity between IFN-γR1 and IFN-γR2 is impaired. Dominant negative mutations have also been identified in *IFNGR2*, somewhat similar to those in *IFNGR1* (Rosenzweig et al., 2004). Vogt et al. (2005) identified a gain of glycosylation site in IFNGR2 leading to impaired signaling. They went on to identify numerous other genetic disorders characterized by aberrant gains of glycosylation, indicating novel pathways of therapy. New forms of partial and complete recessive IFN-γR2 deficiency were subsequently identified (De Paus et al., 2011; Vogt et al. 2008).

IL12B

IL-12B (IL-12p40) combines with IL-12A (IL-12p35) to form the biologically active molecule IL-12p70. IL-12p40 can also function as a homodimer, but in this context it has much less stimulatory activity and may inhibit IL-12 signaling. IL-12B can also combine with p19 to form the biologically active compound IL-23. Complete IL-12B (IL-12p40) subunit deficiency was first described in a child born to consanguineous Pakistani parents who was immunized with BCG at birth (Altare et al., 1998c). Sequencing of *IL12B* revealed a large deletion involving two coding exons resulting in a frameshift deletion of 374 nucleotides between positions 482 and 854 (Fig. 35.1). The parents and a healthy sibling were carriers of this mutation; the affected child was homozygous. Eleven additional patients from five other families have been identified to date (Fieschi and Casanova, 2003; Picard et al., 2002). One child had only salmonellosis. All other patients had mycobacterial disease, BCG-osis in 10 children. Four children with BCG-osis also had salmonellosis, one had tuberculosis, one had *M. chelonae*, and one had nocardiosis. Five children died, but all survivors are well and no longer on treatment. Interestingly, one kindred from India had the same large deletion previously reported in the Pakistani kindred. A founder effect was documented and dated to approximately 29 generations ago (95 percent confidence interval [CI] 9–115) and 700 years ago (95 percent CI 216–2760) by means of a novel mutation-dating method (Genin et al., 2004). The other four kindreds originated from the Arabic peninsula and were all found to carry the same *IL12B* frameshift insertion (315insA) (Picard et al., 2002). A founder effect was again documented and dated to 47 generations ago (95 percent CI 22–110) and 1,100 years ago (95 percent CI 528–2640). The fact that all patients with IL-12B deficiency identified to date have *IL12B* mutations resulting from a founder effect, one in the Indian subcontinent and another in the Arabic peninsula, is consistent with the rarity of IL-12p40 deficiency among patients with MSMD. It was the first example of a founder effect among Mendelian mycobacterial susceptibility genes. Because IL-12p40 is also a component of IL-23, IL-12p40 deficiency results in IL-23 deficiency. IL-12B polymorphisms are clearly associated with a variety of infectious and inflammatory diseases, including psoriasis (Nair et al., 2008), hepatitis C (Houldsworth et al., 2005), cytomegalovirus infection following kidney transplant (Hoffmann et al., 2009), and tuberculosis (Morris et al., 2011).

IL12RB1

IL-12Rβ1 serves as one of the receptor chains for IL-12p70 when it partners with IL-12Rβ2. When it partners with IL-23R, it serves as the receptor for the dimer IL-12p40 and p19, otherwise known as IL-23. Mutations in *IL12RB1* (which encodes the β1 subunit of the IL-12 receptor) have been identified in 141 patients from over 100 families from around the world (Altare et al., 1998a, 2001; Asku et al., 2001; de Beaucoudrey et al., 2010; de Jong et al., 1998; Fieschi et al., 2003; Gruenberg et al., 2010; Sakai et al., 2001; Staretz-Haham et al., 2003; Verhagen et al., 2000; Yancoski et al., 2009). Most patients had BCG or NTM disease, often with salmonellosis, but several were found to suffer from salmonellosis only and some from tuberculosis only (de Beaucoudrey et al., 2010; Fieschi et al., 2003; Fieschi and Casanova, 2003; Sanal et al., 2006; Staretz-Haham et al., 2003). One case of visceral leishmaniasis has been reported as well (Sanal et al., 2007). *Klebsiella* infection has also been reported in IL-12Rβ1 deficiency (Pedraza et al., 2010), as has esophageal cancer and mucocutaneous candidiasis (Cardenes

et al., 2010). A significant fraction of patients were asymptomatic. A total of 34 unique mutations have been identified, including nonsense, splice, and frameshift mutations, which lead to premature termination of translation in the extracellular domain (Fig. 35.1), abrogating cell surface expression of IL-12Rβ1 resulting in complete deficiency. Missense mutations resulting in a lack of receptor expression at the cell surface have been validated as well (Altare et al., 2001; Sakai et al., 2001). Complete IL-12/IL-23 receptor β1 deficiency due to cell-surface–expressed nonfunctional receptors also occurs (Fieschi et al., 2004). Only recessive, loss-of-function mutations have been identified in *IL12RB1* to date. *IL12RB1* mutations also impair IL-23 activation, as shown by the impaired induction of IL-17–producing cells by IL-12Rb1–deficient lymphocytes (de Beaucoudrey et al., 2008).

STAT1

The identification of two unrelated families presenting with MSMD in the absence of mutation in any of the genes discussed above led to the discovery of the fifth MSMD gene. A 33-year-old French woman with a history of disseminated BCG infection following childhood vaccination and a 10-year-old North American girl with disseminated *M. avium* infection were found to carry de novo mutations (the parents of both patients had two wild-type copies of *STAT1*) in the coding region of *STAT1* (Dupuis et al., 2001). Both were heterozygous for c.2116T>C resulting in L706S in the COOH-terminal region (Fig. 35.1). The abnormal protein exerts a dominant negative effect on the normal protein in response to IFN-γ, which leads to STAT-1 dimer formation (GAF), but not in response to IFN-α, which leads to formation of STAT-1/STAT-2/p48 trimers (also known as interferon-stimulated gamma factor 3 [ISGF3]). The *STAT1* mutation is remarkable because it is dominant for IFN-γ signaling (it impairs phosphorylation of tyrosine 701 leading to impaired GAF activation) but recessive for ISGF3 activation. To our knowledge it is the first reported mutation in a human gene to be dominant and recessive for two cellular phenotypes. Vulnerability to mycobacteria and resistance to viruses indicate that GAF mediates antimycobacterial IFN-γ activity, whereas the antiviral effects of IFNs are either STAT-1 independent or ISGF3 dependent. This novel disorder proves that IL-12–induced, IFN-γ–mediated immunity against mycobacteria is both STAT-1 and GAF dependent.

In contrast to the effect of the heterozygous *STAT1* mutation L706S, homozygous loss-of-function mutations lead to loss of IFN-α/β and IFN-γ ability to activate STAT1-containing transcription factors. Two unrelated infants found to be homozygous for two unique mutations of *STAT1* in exon 20 (Fig. 35.1) suffered from mycobacterial disease, but in the complete absence of STAT1 activity both died of viral disease (Dupuis et al., 2003). Furthermore, viral multiplication in vitro was not inhibited by adding recombinant IFN-α/β to cell lines from the two individuals. Thus, complete impairment of STAT1-dependent responses to human IFN-α/β results in increased susceptibility to both mycobacterial and viral diseases (Chapgier et al., 2006a; Dupuis et al., 2003). Subsequently, several more mutations in STAT1 have been identified, some working as autosomal recessive partial defects of IFN-α/β and IFN-γ signaling (Chapgier et al., 2009; Kong et al., 2010b; Kristensen et al., 2010) and others working as autosomal dominant inhibitors of GAF formation (Averbuch et al., 2011; Chapgier et al., 2006b). More recently, gain-of-function mutations in STAT1 have been associated with chronic mucocutaneous candidiasis, autoimmunity, epithelial cancers, and aneurysms (Liu et al., 2011; van de Veerdonk et al., 2011). Another cohort of patients with the same mutations predominantly in the coiled-coil domain have localized nodal mycobacterial disease along with disseminated coccidioidomycosis and histoplasmosis (Sampaio et al., 2013).

NEMO

The identification of boys with osteopetrosis, lymphedema, ectodermal dysplasia, and severe immune deficiency who were born to mothers with incontinentia pigmenti led to the identification of the syndrome now known as NEMO deficiency (Doffinger et al., 2001; Jain et al., 2001) (see Chapter 36). NEMO, the NFκB essential modulator, is critical to the signal transduction from numerous cell-surface Toll-like receptors and TNF family receptors (Shih et al., 2011). NEMO (also known as IKKγ) is required for the integrity of the kinase complex that phosphorylates IκB, the inhibitor that keeps NFκB tethered in the cytoplasm and unable to transactivate target genes. Activation of the IκB kinase (IκK, composed of α, β, and γ subunits) leads to ubiquitination and phosphorylation of IκB, liberating NFκB (Gautheron and Curtois, 2010). NEMO is central to several signal transduction pathways, including most TLR receptors, IL-1 receptor, TNF-α receptor, and dysplasin, a receptor associated with isolated ectodermal dysplasia, explaining the complex syndrome that is NEMO deficiency. Filipe-Santos et al. (2006b) showed in several families that NEMO accounted for one form of X-linked susceptibility to mycobacterial disease. Hanson et al. (2008) performed an exhaustive survey of reported mutations and suggested broad possible genotype–phenotype associations. However, it is clear that the syndrome is quite variable and that the somatic and immunological features can be easily segregated in a given case. Therefore, there is no requirement that any particular patient with NEMO immunodeficiency has ectodermal dysplasia (Hoshina et al., 2011).

CYBB

gp91^{phox} encoded by *CYBB* is a major component of the NADPH oxidase (NOX2) complex that traverses the cytoplasm or phagosome membrane in complex with its partner, p22^{phox}. With phagocyte activation, cytoplasmic proteins complex to the gp91^{phox}/p22^{phox} heterodimer and facilitate the oxidation of NADPH with the coincident generation of superoxide. Mutations in any of the five genes involved in the NADPH oxidase lead to the condition known as chronic granulomatous disease (CGD) (Segal et al., 2000) (see Chapter 52). Typically, patients with CGD have a host of

infections and inflammatory problems, usually beginning in infancy or childhood. Mutations in *CYBB* leading to impaired superoxide production are typically manifest in neutrophils, monocytes, macrophages, and B cells and detected by assays of cellular superoxide generation in response to stimulation. Mycobacterial infections, especially those due to BCG and tuberculosis, are part of the spectrum of CGD infections (Bustamente et al., 2007a; Winkelstein et al., 2000). In infancy severe BCG infections are well recognized to be increased in CGD and often precede other infections. Bustamente et al. (2007b) recognized a syndrome that appeared X-linked based on inheritance and manifest with only extrapulmonary BCG and tuberculosis. Subsequently, they showed that this X-linked gene is *CYBB* (Bustamante et al., 2011a, 2011b). This was quite surprising since these patients have normal neutrophil and monocyte gp91^{phox} protein expression and superoxide production. However, differentiated macrophages and Epstein-Barr virus (EBV)-transformed B cells display a much more profound defect of gp91^{phox} expression, resulting in impaired gp91^{phox}/p22^{phox} heterodimer assembly and impaired superoxide production (Bustamente et al., 2011a, 2011b). The mutations causing this condition are novel and are located in the transmembrane domain and extracellular loop, indicating that regulation of gp91^{phox} expression is tightly regulated at the differentiated cell level and that not all mutations in *CYBB* are detectable in neutrophils and monocytes.

IRF8

Two types of lRF8 deficiency have been recently reported (Hambleton et al. 2011). In a child born to consanguineous parents, disseminated BCG disease led to the discovery of a lack of circulating monocytes and dendritic cells in infancy. The severity of disease, mimicking severe combined immunodeficiency, required hematopoietic stem cell transplantation. Following a candidate gene approach, the child was found to have complete IRF8 deficiency due to a homozygous missense change (K108E). IRF8 is expressed at high levels in myeloid cells and mediates interferon and TLR responses, indicating that the IL-12/IFNγ circuit would be profoundly disrupted by an IRF8 defect. In two additional, unrelated patients with a history of recurrent BCG infection, an identical heterozygous IRF8 (T80A) mutation was found, defining an autosomal dominant partial form of IRF8 deficiency. The mutation is severely hypomorphic and dominant negative. Interestingly, the IL-12-IFNγ circuit does not appear to be disrupted. Nevertheless, the population of circulating CDlc^{+} CD11c^{+} myeloid dendritic cells is lacking in the two patients with autosomal dominant lRF8 deficiency. This cell population produces high amounts of IL-17 in normal individuals, indicating that it is possible that their absence may contribute to these patients' MSMD phenotype.

GATA2

The syndrome of monocytopenia, B and NK lymphocytopenia, and mycobacterial infections has been recognized for several decades, in some cases associated with myeloid cancers such as acute myelogenous leukemia (AML) or chronic myelomonocytic leukemia (CMML) as well as myelodysplasia (MDS) (Holland et al., 1994; reviewed in Vinh et al., 2010). This syndrome has been called MonoMAC for its concurrent monocytopenia and mycobacterial infection (Vinh et al., 2010) or DCML for its dendritic cell, monocyte, and B and NK lymphoid deficiency (Bigley et al., 2011). Hahn et al. (2011) identified GATA2 as the gene responsible for familial AML and MDS, leading to its investigation in MonoMAC/DCML. Hsu et al. (2011) sequenced GATA2 in their cohort of MonoMAC patients and found 12 distinct heterozygous missense and insertion/deletion changes in 20 patients, demonstrating that GATA2 haploinsufficiency was the cause of the syndrome. Dickinson et al. (2011) used whole-exome sequencing to find missense changes in the second zinc finger of GATA2 in four patients with the same disease. Subsequently, GATA2 was show to be the etiologic gene for the syndrome of lymphedema and MDS/AML, known as Emberger syndrome (Ostergaard et al., 2011). GATA2 has extensive effects on the early development of lymphatics and their valves that explain its role in Emberger syndrome (Kazenwadel et al., 2012). GATA2 is a transcription factor expressed in early hematopoiesis but also expressed in many terminally differentiated hematologic and somatic cells. The clinical presentation of GATA2 deficiency is remarkable for relatively late onset (late childhood to midlife) with disseminated NTM, histoplasmosis, and warts. There have been no cases of BCG infection so far, which may reflect lack of exposure but is more likely due to relatively intact hematopoiesis early in life that slowly fails over time. The precise mechanism by which GATA2 deficiency leads to NTM susceptibility is unknown, but the lack of monocytes and NK cells removes important responders to and producers of IFNγ.

ISG15

Two unrelated patients, a 15 year old girl and a 12 year old boy with unexplained MSMD, both born to consanguineous parents, were investigated by combined whole-exome sequencing and genome-wide linkage analysis. Two disease-causing homozygous mutations, one a nonsense and one a frame shift mutation in the *ISG15* gene were identified, resulting in undetectable protein in both patients (Bogunovic et al., 2012). ISG15 is an intracellular IFN-α/β-inducible protein that conjugates to proteins in an ubiquitin-like fashion (Sauk et al., 2010) and is known to be a potent IFN-γ-inducing cytokine playing an essential role in anti-mycobacterial immunity. The clinical and immunological phenotypes of these two ISG15-deficient patients resemble those of patients with IL-12p40 or IL-12Rβ1 deficiency, with impaired but not abolished IFN-γ immunity and relatively mild MSMD.

MUTATION ANALYSIS

Mutations in *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1* (Fig. 35.1), *IRF8*, *NEMO*, *CYBB*, and *GATA2* can be detected by direct sequencing. Primers to amplify and sequence all

exons and flanking intron regions have been published and are available upon request.

STRATEGIES FOR DIAGNOSIS

CLINICAL DIAGNOSIS

Inherited defects of the IL-12/IFN-γ axis (and those of IRF8, NEMO, CYBB, and GATA2) should be considered in the differential diagnosis of all patients presenting with severe infection (including disseminated and recurrent diseases) with intracellular microorganisms, particularly when the organism is considered to be nonpathogenic in the "immunocompetent" individual. However, these defects should be sought especially aggressively in patients with severe nontuberculous mycobacterial or *Salmonella* infections. Furthermore, a high index of suspicion is warranted in patients presenting with chronic fever, wasting, hepatosplenomegaly, lymphadenopathy, and anemia in whom a pathogen is not isolated, as cultures may be persistently negative (Levin et al., 1995; Pierre-Audigier et al., 1997). Diagnosis may also be confounded by the lack of usually diagnostic granulomata, in which microbes may or may not be visible. An initial diagnosis of histiocytosis X has occasionally been made, so MSMD should be considered in chemotherapy-resistant children with a tentative diagnosis of histiocytosis without formal histological criteria (Edgar et al., 2001). In many individuals, MSMD becomes apparent following BCG vaccination, so vaccination history is essential. Because of the high incidence of parental consanguinity and affected relatives, family history is important. Specific attention must be directed at possible parental consanguinity. Defects in the IL-12/IFN-γ axis should not only be sought in patients with disseminated or recurrent BCG/NTM disease but also considered in patients with acute local BCG/NTM, severe tuberculosis, severe histoplasmosis, listeriosis, paracoccidioidomycosis, coccidioidomycosis, and severe viral infections. Despite the progress outlined in this chapter, our understanding of the molecular basis of MSMD is still in its relative infancy; it is likely that there are many aspects of the disease that are yet to be unraveled. It is therefore prudent to consider the disorder in those diagnostic conundrums in which infectious, malignant, or inflammatory diagnoses are entertained.

LABORATORY DIAGNOSIS

In vitro testing is based on (1) circulating IFN-γ levels, (2) protein expression (FACS, ELISA), (3) functional studies, and (4) DNA analysis.

Levels of circulating IFN-γ in either plasma or serum are frequently abnormal in actively infected patients with complete IFN-γR deficiency (Fieschi et al., 2001). These children have high levels of plasma IFN-γ, whereas IFN-γ is low or undetectable in plasma taken from healthy controls. Patients with partial IFNγR1 deficiency typically have lower levels of circulating IFN-γ (Soluguren et al., 2011). High IFN-γ levels have been thought to be due to sustained production of IFN-γ in the setting of active disease, along with the requirement for an intact IFN-γR for ligation and removal of IFN-γ from the circulation. Although this observation provides a simple assay for individuals with severe BCG/NTM disease who are suspected to have defects in the IFN-γR, it should be kept in mind that elevated plasma or local (e.g., pleural tuberculosis) IFN-γ levels are seen in normal hosts with active disease. Further, when patients with complete IFN-γR deficiency are not actively infected, their circulating IFN-γ levels are normal. Similar data have been clearly shown in mouse models (Rottman et al., 2008). In addition, normal ranges for circulating IFN-γ levels in the setting of other diseases (bacterial osteomyelitis, leukemia) are not available, making interpretation of this test complex at best. Therefore, while an elevated IFN-γ level may be useful in suggesting a complete IFN-γR defect, it is not shown to be helpful in excluding or including other defects in the IFN-γ/IL-12 circuit.

The IFN-γR is expressed ubiquitously on all nucleated cells, whereas the IL-12 receptor is found only on NK and T cells. Analysis of EBV-induced B lymphoblasts, SV-40 fibroblasts, or peripheral blood mononuclear cells (PBMCs) for IFN-γR cell-surface expression by fluorescence-activated cell sorting (FACS) is a simple means to assess the presence or absence of these receptors, as demonstrated for the first reported cases of complete IFN-γR1 deficiency (Jouanguy et al., 1996; Newport et al., 1996). Mutations causing the dominant form of IFN-γR1 deficiency abrogate the receptor recycling motif, leading to high levels of cell-surface IFN-γR1 expression (up to 10-fold), which is easily detectable by FACS staining (Dorman et al., 2004; Jouanguy et al., 1999b; Sasaki et al., 2002; Villela et al., 2001). Normal expression of IFNγ-R1, as detected by FACS, even using blocking antibodies, does not exclude partial or even complete IFNγ-R1 deficiency from mutations that result in the surface expression of an abnormal protein (Jouanguy et al., 1997, 2000). Antibodies that recognize low levels of IFN-γR2 present on resting cells are not yet adapted to routine laboratory use. Antibodies that recognize IL-12Rβ1 work well on phytohemagglutinin (PHA) blasts and allow a diagnosis of IL-12Rβ1 deficiency. To date, most *IL12RB1* mutations identified cause a loss of expression of the encoded chain. There is one mutation with residual expression of IL-12Rβ1 (de Beaucoudrey et al., 2010; Fieschi et al., 2004). Staining of *Herpesvirus saimiri*-transformed T-cell lines and EBV-transformed B lymphoblasts also works well. Secreted IL-12p40 and p70 can be detected by ELISA in the supernatant of whole blood or PBMCs stimulated by BCG or IFN-γ as well as in the supernatant of EBV-transformed B lymphoblasts stimulated with phorbol 12,13-dibutyrate (PDBu) (Feinberg et al., 2004; Picard et al., 2002). To date, all *IL12B* mutations identified have been associated with a lack of detectable IL-12p40 and IL-12p70. However, prolonged stimulation of IL-12Rβ1–deficient T-cell lines has been reported in one instance to rescue some degree of IL-12 responsiveness (Verhagen et al., 2000).

Expression of IFN-γR or IL-12R does not necessarily confer function, and therefore other in vitro assays are required to identify which component(s) of the IL-12/IFN-γ pathway are defective in appropriate cases. Upon binding to its receptor, IFN-γ induces pleiotropic effects, including the upregulation of major histocompatibility complex (MHC) class II expression and TNF-α production by monocytes. These effects are mediated by the binding of phosphorylated STAT1

to gamma-activating sequences (GAS) in gamma-responsive genes. Thus, IFN-γR deficiency may be diagnosed functionally by studying in vitro responses to IFN-γ. A simple whole-blood assay was used to demonstrate defective responses to IFN-γ in the Maltese kindred (Levin et al., 1995), and this technique was modified to study PBMC responses in a patient with partial IFN-γR1 deficiency (Jouanguy et al., 1997). MHC class II expression is easily studied by flow cytometry (Altare et al., 1998b), whereas nuclear translocation and phosphorylation of pSTAT1 in response to IFN-γ can be assessed using electrophoretic mobility shift assays (Bach et al., 1997; Dupuis et al., 2001) or, more simply, by flow cytometry with a STAT1-specific monoclonal antibody (Fleisher et al., 1999). Cellular responses to IFN-γ should be tested at low and high concentrations of IFN-γ to differentiate between partial and complete receptor deficiency. In vitro studies in patients with IL-12p40 deficiency show defective IFN-γ production by PBMCs or whole blood following stimulation with BCG. This defect was restored by the addition of recombinant IL-12 to the culture medium. IL-12Rβ1–deficient patients also have diminished mitogen-induced IFN-γ production, but IL-12 p70 production in response to lipopolysaccharide (LPS), tuberculin, or mycobacteria is normal. A flow-cytometric assay that detects phosphorylated STAT4 has also been developed (Uzel et al., 2001). Notably, children with complete IFN-γR deficiency have low in vitro production of IFN-γ because of impaired production of IL-12 (Holland et al., 1998).

Recurring mutations are seen in the dominant *IFNGR1* hot-spot mutations, IRF8 hot-spot mutations, GATA2 hot-spot mutations, some recessive hypomorphic IFNGR1 mutations, and the two *IL12B* mutations (founder effects). The majority of the rest of the MSMD mutations are unique. It is not cost-effective to set up mutation screening assays looking only for known mutations. A combination of in vitro phenotyping (flow cytometry for surface expression of the IFN-γR1 and IL-12Rβ1 and functional studies) followed by direct gene sequencing is recommended. GATA2 deficiency is suggested by markedly low numbers of circulating monocytes, B cells, and NK cells. Once the causative mutation has been established in a family, other family members can be screened directly for the mutation. Accurate molecular diagnosis by biochemical, functional, and genetic studies is of the utmost importance for guiding treatment and predicting prognosis.

GENETIC COUNSELING AND PRENATAL DIAGNOSIS

Defects in the IL-12/IFN-γ pathway may be inherited either as dominant or recessive disorders, depending on the mutation. All mutations reported in *IFNGR2*, *IL12RB1*, and *IL12B* are recessive: many patients are homozygous, reflecting the high frequency of parental consanguinity within this group of patients. *IFNGR1* mutations were initially identified as homozygous recessively inherited traits, but dominant mutations have subsequently been identified as well. Compound heterozygotes have also been reported. Most *STAT1* mutations identified to date are dominant loss- or gain-of-function mutations, but complete recessive cases have also been recognized. Mutations in IRF8 are recessive or dominant, while those in GATA2 are transmitted in a dominant pattern but cause disease because of haploinsufficiency. Finally, X-linked recessive inheritance has been shown to be due to mutations in NEMO (Frucht and Holland, 1996; Frucht et al., 1999; Holland et al., 1994) and more recently in gp91^{phox} (NOX2) (Bustamante et al., 2011a).

Given the heterogeneity of MSMD, coupled with its rarity, carrier detection or screening using functional assays is not currently feasible. In one family with recessive IFN-γR1 deficiency, heterozygous carriers had an intermediate cellular phenotype in vitro (Levin et al., 1995; Newport et al., 1996), although this may have been dependent on the assay used. To date, there is no clinical phenotype associated with heterozygosity for any of the recessive alleles. Once the molecular basis is known within a family, it is simplest to screen other members by directly sequencing their DNA. Counseling within families in which the mutation is known is straightforward in terms of the risk of inheriting a "susceptible" genotype (25 percent risk of an affected child if recessive, 50 percent risk of an affected child if dominant inheritance). However, any discussion must also take into account the following: (1) the clinical phenotype depends on the gene affected and whether the mutation leads to complete or partial protein deficiency; (2) development of disease is dependent on pathogen exposure (e.g., BCG); and (3) there are individuals who have inherited a susceptible genotype without developing disease, presumably thanks to the impact of modifier genes that result in residual antimycobacterial immune function or lack of exposure or both. To date, there are no known individuals with complete IFN-γR1, IFN-γR2, IRF8, or STAT1 deficiency who have not been clinically affected. Complete IFN-γR1 or IFN-γR2 deficiency is the most severe phenotype and is frequently lethal despite the use of antibiotics. BCG vaccination must be withheld from potentially affected children until IFN-γR status is clarified. Bone marrow transplantation (BMT) has proved very difficult and less successful than would be anticipated (see below), perhaps because transplantation has typically been attempted after disseminated mycobacterial disease occurred.

Once a molecular diagnosis has been established, prenatal diagnosis can be offered to affected families with severe disease (i.e., complete IFN-γR deficiency). The role of prenatal diagnosis for other mutations is less obvious as the phenotype is less severe, disease is preventable, and many individuals carrying mutations are disease-free.

TREATMENT AND PROGNOSIS

The treatment of defects in the IL-12/IFN-γ axis should be tailored to the individual patient according to the mutation, the clinical pattern of disease, and the pathogens involved (Holland, 2000b). Established infection should be treated with appropriate antimicrobial drugs as determined by the genus and species. Thus, microbiological isolation and characterization of the causative pathogen at an early stage are desirable. The role for in vitro susceptibilities in directing treatment of NTM infections is still unproven and poorly defined.

NTM infections are notoriously resistant to a number of antimicrobials. Cytokine therapy has helped clear mycobacterial infection in patients with full or partial function of the IFN-γ receptor (Holland, 2000a; Holland et al., 1994), and patients with IL-12B (IL-12p40), IL-12Rβ1 deficiency or partial STAT1 deficiency respond well to IFN-γ treatment. It may also work in patients with the dominant IRF8 mutations and the mycobacteria-predisposing mutations in gp91^{phox} (NOX2). However, intestinal, mesenteric, and splenic infections can be resistant to antibiotics and IFN-γ. Splenectomy was helpful in two children with splenic sequestration (IFN-γ induced in one child); on occasion abdominal lymph node resection may be indicated (Kaufman et al., 1998; J. L. Casanova, unpublished observation). Overall, patients with partial IFN-γR/STAT1 deficiency or complete IL-12Rβ1 deficiency can achieve prolonged clinical remission after antibiotics and IFN-γ are discontinued. Relapses may occur years after the initial episode. Treatment with antibiotics and IFN-γ should be prolonged, even after clinical remission is obtained.

In contrast, children with complete IFN-γR deficiency achieve full clinical remission less often, and mycobacterial infections often relapse weeks to months after antibiotics are discontinued (Dorman et al., 2004). Therefore, successful antibiotic therapy should not be discontinued. Because of the lack of specific receptors, IFN-γ therapy is not indicated. The role for other cytokines, such as IFN-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-12, is undefined. The only curative treatment available for patients with complete IFN-γR deficiency is BMT (Reuter et al., 2002; Chantrain et al., 2006; Moilanen et al. 2009). An international survey identified eight unrelated patients with complete IFN-γR1 deficiency who underwent BMT (Roesler et al., 2004). The results were disappointing: BMT in patients with complete IFN-γR1 deficiency is associated with high morbidity and mortality. The only child receiving a human leukocyte antigen (HLA)-haploidentical transplant died after the second transplant (HLA identical) of EBV-induced lymphoproliferative disease. Of the seven patients transplanted with an HLA-identical related graft, despite an initial full engraftment in most cases, four are alive but only two have a functional graft and are free of infection. There appears to be a selective advantage of IFN-γR–deficient over wild-type hematopoietic progenitors in IFN-γR–deficient children (which makes gene therapy for patients with IFN-γR deficiency challenging) (Rottman et al., 2008). Moreover, chronic NTM/MAC infection before and during stem cell transplantation carries an unfavorable outcome. The expression of IFN-γ itself appears to inhibit marrow engraftment and success of transplant, which is greatly aggravated in the setting of ongoing active infection. Prevention of infection is desirable, although many pathogens to which these individuals are susceptible are ubiquitous in the environment. BCG should be avoided and mycobacterial infection (both primary and secondary) may be prevented by the routine daily use of a macrolide such as clarithromycin or azithromycin. In patients with mild MSMD, prophylactic antibiotics are not required, as infectious episodes are relatively infrequent and can be controlled by IFN-γ and antibiotics if treated promptly. However, physicians and patients should weigh carefully the risks and benefits of recurrence of infection, especially if it recurs in bone, as is often the case with the dominant form of IFN-γR1 deficiency (Dorman et al., 2004). In these patients recurrence of infection can have serious consequences, despite curative therapy.

In patients with complete IFN-γR deficiency, antibiotics should be continued indefinitely after therapy for acute infections. There is considerable diversity of pathogenic NTM (particularly rapidly growing species), making absolute recommendations difficult. Most NTM are susceptible to macrolides, and these should be strongly considered for long-term prophylaxis regardless of cure of other acute infections. Immunosuppressive agents such as corticosteroids should be avoided as a rule, particularly in children with complete IFN-γR deficiency, although in some circumstances they may be helpful. Children with MSMD should be treated on an individual basis, and treatment should be undertaken in close collaboration with experts.

ANIMAL MODELS

The study of gene-disrupted mice has greatly enhanced our understanding of the IL-12/IFN-γ pathway. Although not completely concordant, the phenotypic similarities between these animal models and patients with mutations in this axis are striking (Jouanguy et al., 1999a). Mice lacking *Ifngr1* are highly susceptible to BCG infection, with poorly defined granuloma formation and death (Kamijo et al., 1993). Mice lacking Ifn-γ also fail to control BCG, *M. avium*, or *M. tuberculosis* (Cooper et al., 1993; Dalton et al., 1993). More recently, *Ifngr2* knockout mice were shown to have defective Ifn-γ production and susceptibility to *L. monocytogenes* infection (Lu et al., 1998). *Il12b* (il-12p40) knockout mice are more susceptible to *M. tuberculosis* infection than normal mice, leading to higher bacterial loads and disseminated disease (Cooper et al., 1997). Granulomata were poorly formed and multibacillary. *Il12rb1* knockout mice have defective Ifn-γ responses to mitogens and LPS (Wu et al., 1997). Disruption of *Ifng* in mice also leads to lethal infection with an attenuated strain of *S. typhimurium*, whereas wild-type mice clear infection within 4 weeks (Bao et al., 2000; Hess et al., 1996). However, comparisons between mice and humans are limited in several ways: most of the infections in MSMD patients are naturally occurring, whereas those in mice are experimental and often administered intravenously, and the strain and dose of pathogen are controlled. There are certain infections, such as *Toxoplasma gondii* and *Cryptococcus neoformans*, to which *Ifng/Il-12* knockout mice have increased susceptibility that are less observed in humans (Decken et al., 1998; Yap et al., 2000). This may reflect lack of exposure, experimental design, or the fact that knockout mice are generated in highly inbred strains. Genetic variation at other immunity-modifying loci is low in inbred mice whereas humans are outbred, even in the setting of consanguinity. Experimental infections in mice probably highlight even minor effects of the Il-12/Ifn-γ axis. Alternatively, mice and humans may differ in their handling of some of these nonmycobacterial infections.

CONCLUDING REMARKS AND FUTURE CHALLENGES

Mutations in eight genes involved in the IL-12/IFN-γ axis, the NADPH oxidase pathway, or early hematopoiesis have been associated with the syndrome of MSMD, which encompasses a range of clinical presentations. The severity of the clinical phenotype depends primarily on the gene involved and the specific mutation. IFN-γ–mediated immunity appears to be a genetically controlled quantitative trait that determines the outcome of mycobacterial invasion (Dupuis et al., 2000). IFN-γ immunity to mycobacteria is dependent on IL-12 stimulation and mediated by STAT1 and its homodimeric complex GAF. These defects are most pronounced with respect to mycobacteria and, to a lesser extent, intramacrophagic fungi, *Salmonella*, and viruses (Casanova and Abel, 2002). The investigation of more patients is necessary to broaden our knowledge of these genotype–phenotype correlations. Clinically, molecular diagnosis guides rational treatment based on pathophysiology.

ARE THERE OTHER MSMD GENES?

There remain patients with the clinical syndrome of MSMD who do not have mutations in *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1, NEMO, CYBB, IRF8*, or *GATA2* (approximately 20 percent at our centers, S. Holland and J. L. Casanova, unpublished observations). Characterization of the molecular defects in these patients will help in the identification of other MSMD genes and contribute further to our understanding of human mycobacterial immunity. Relevant genes upstream of IL-12 and downstream of IRF8 are expected to expand and define the limits of the IL-12/IFN-γ axis, especially the inducer and effector mechanisms of immunity to mycobacteria.

DEFINITION OF CLINICAL BOUNDARIES OF MSMD

The genetic defects of the IL-12/IFN-γ axis were identified by investigating patients with disseminated, often lethal, BCG/NTM disease. Subsequently, it was found that some affected individuals have recurrent local disease, whereas others are asymptomatic. The clinical boundaries of IL-12Rβ1 deficiency (de Beaucoudrey et al., 2010; Fieschi et al., 2003) and IFN-γR1 deficiency (Dorman et al., 2004; Solugren et al., 2011) were made possible thanks to international collaboration. Other international surveys are currently under way to define the clinical features of each inherited disorder on the basis of the clinical history of the patients identified. The question arises as to whether patients with unexplained local BCG/NTM disease may suffer from these or related genetic defects. However, NTM lung disease in the elderly appears to be due to other predisposing factors and does not appear to be due to impaired hematopoietic IFN-γ synthesis or response. NTM adenitis in childhood is currently unexplained but does not appear to have its origin in immune defects in this pathway (Haverkamp et al., 2010). Because of these considerations, patients with various forms of BCG/NTM disease should be studied in terms of the IL-12/IFN-γ axis to define the clinical frontiers of each genetic defect.

ROLE OF MSMD GENES IN SUSCEPTIBILITY TO TUBERCULOSIS AND LEPROSY

It is estimated that approximately 2 billion individuals worldwide are infected with *M. tuberculosis* (Dolin et al., 1994). The World Health Organization estimates that there were 8 million new cases of tuberculosis and 1.7 million deaths from the disease in 2009. The fact that only 10 percent of individuals infected with *M. tuberculosis* go on to develop clinical disease suggests that exposure to virulent mycobacteria alone is not sufficient and that the host immune response is an important determinant of susceptibility (or resistance) to disease (Murray et al., 1990). Several studies demonstrate a role for host genetic factors as determinants of susceptibility to tuberculosis (Casanova and Abel, 2002, 2004). However, the identification of specific genes involved in susceptibility to infectious diseases in outbred human populations is difficult. Complex interactions among the pathogen, which also has a genome, the environment, and host factors determine whether an individual is resistant or susceptible to disease. It is likely that a number of genes are involved, but it is not known exactly how many or how they interact. Population-based studies have reported associations between candidate genes and tuberculosis, but the effects have been modest and the functional relevance of these findings is yet to be established (Abel and Casanova, 2000; Casanova and Abel 2002, 2004; Newport and Levin, 1999; Wilkinson et al., 1999).

There is a spectrum of disease within the MSMD syndrome ranging from severe disease that is fatal in early childhood (complete IFN-γR deficiency) to moderate disease in individuals with partial IFN-γR1 deficiency (Jouanguy et al., 1997). The IL-12p40/IL-12Rβ1 mutations have a less severe clinical course. Mutations in *IL12RB1* and *IL12B* have been identified as a susceptibility factor for the development of abdominal *M. tuberculosis* infection (Altare et al., 2001; Haerynck et al., 2008) and tuberculous adenitis (Picard et al., 2002). Furthermore, four families have been observed whose affected members lacked IL-12β1 and suffered from disseminated tuberculosis; however, none of them had a history of clinical disease caused by BCG/NTM (Altare et al. 2001; Boisson-Dupuis et al., 2011; Caragol et al., 2003; Özbeck et al., 2005). Disorders of the IL-12/IFN-γ axis should thus be considered in selected children with severe tuberculosis, even in the absence of infection with poorly virulent mycobacteria or *Salmonella*. Partial deficiency of either IL-12p40 or IL-12Rβ1 would be expected to have a less severe phenotype than complete deficiency and to show susceptibility to only the most virulent pathogens. More subtle polymorphisms in the MSMD genes identified thus far could result in impaired expression of a normal protein or normal expression of a slightly altered, less efficient protein. It is also likely that mutations or polymorphisms will be identified in other genes known to be involved in mycobacterial immunity that may play a different role and cause a different immune defect. Such individuals may retain immunity to organisms of low virulence while remaining susceptible to more virulent species.

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36.

INBORN ERRORS OF NF-κB IMMUNITY: GENETIC, IMMUNOLOGICAL, AND CLINICAL HETEROGENEITY

Capucine Picard, Jordan S. Orange, Anne Puel, Shen-Ying Zhang, and Jean-Laurent Casanova

The 10 human Toll-like receptors (TLRs) act as receptors for pathogens and, as such, are involved in innate immune recognition (Akira and Takeda, 2004). Agonists of TLR homodimers and heterodimers include triacetylated bacterial lipopeptides for TLR1/TLR2, diacetylated bacterial lipopeptides for TLR2/TLR6, peptidoglycan and lipoteichoic acid for TLR2, double-stranded RNA for TLR3, lipopolysaccharide (LPS) for TLR4, bacterial flagellin for TLR5, single-stranded RNA for TLR7 and TLR8, and CpG-DNA for TLR9 (Kawai and Akira, 2010). No TLR10 agonist has yet been identified. TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface. By contrast, TLR3, 7, 8, and 9 are expressed in the endoplasmic reticulum (ER), from which they may be transported to other intracellular compartments, such as endosomes. UNC-93B is an essential molecule in the signaling pathway of these receptors because it delivers the nucleotide-sensing receptors TLR3, 7, 8, and 9 from the ER to endolysosomes (Fig. 36.1) (Kim et al., 2008).

TLRs and IL-1Rs all have an intracellular Toll and interleukin-1 receptor (TIR) domain. The presence of this domain defines the TIR superfamily (O'Neill, 2008). Humans have up to 10 members of the IL-1R family, including a decoy receptor without a TIR domain (IL-1RII) and 9 TIR-domain-containing receptors, including 6 receptors (IL-1R1, IL-18Rα, IL-33Rα, TIGIRR-1, TIGIRR-2, and IL-1Rrp2), 2 co-receptors (IL-1RAcP and IL-18Rβ), and an inhibitory receptor TIR8/SIGIRR (Arend et al., 2008; Dinarello, 2009; Sims and Smith, 2010). The six receptors include those for three potent proinflammatory cytokines: (1) IL-1β (IL-1RI and IL-1RAcP also recognize the less-inflammatory IL-1α) (Arend et al., 2008; Dinarello, 2009, Sims and Smith, 2010), (2) IL-18 (IL-18Rα and IL-18Rβ also recognize the less-well-defined IL-1F7) (Arend et al., 2008; Nakanishi et al., 2001), and (3) IL-33 (IL-33Rα and IL-RAcP) (Arend et al., 2008; Haraldsen et al., 2009). Historically, IL-1Rs have been seen as key innate immune system receptors, because IL-1β, IL-18, and IL-33 have been shown to be key cytokines in the early steps of the inflammatory response (Arend et al., 2008; Dinarello, 2009; Sims and Smith, 2010). These three cytokines, IL-1β, IL-18, and IL-33, are thought to play an important role in various immune responses contributing to host defense.

TIR-domain-containing TLRs and IL-1Rs recruit the TIR-containing cytosolic adaptors MyD88, TRIF, TIRAP (also known as MAL), TRAM, and SARM (Kenny and O'Neill, 2008; O'Neill and Bowie, 2007). The canonical TIR pathway is dependent on MyD88, which is used by all TLRs other than TLR3 and at least three IL-1Rs: IL-1R, IL-18R, and IL-33R. The alternative pathway is controlled by another key adaptor, TRIF, the only known TLR3 adaptor, which is also used by MyD88 via TLR4 (Fig. 36.1). The other three adaptors act as co-adaptors. The sorting adaptor TIRAP recruits MyD88 to TLR2 and 4, whereas TRAM recruits TRIF to TLR4. The role of SARM is less well defined (Carty et al., 2006; Casanova et al., 2011). The adaptors, in turn, recruit cytosolic kinases, including the IL-1R–associated kinase (IRAK) complex, which is recruited by MyD88 and seems to be the most TIR-specific kinase used in these pathways (Lin et al., 2010; Ringwood and Li, 2008). The classical TIR pathway results in the activation of both nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), via the IRAK complex, which comprises two active kinases (IRAK-1 and IRAK-4) (Fig. 36.1) and two noncatalytic subunits (IRAK-2 and IRAK-3/M). The alternative pathway results in the activation of NF-κB and interferon regulatory factor 3 (IRF3).

NF-κB is a transcription factor sequestered in the cytoplasm of resting cells through associations with the inhibitor of NF-κB (IκB) proteins. Signaling via both the classical and alternative pathways can lead to the activation of NF-κB. Upon cell stimulation, IκBs are phosphorylated at two conserved

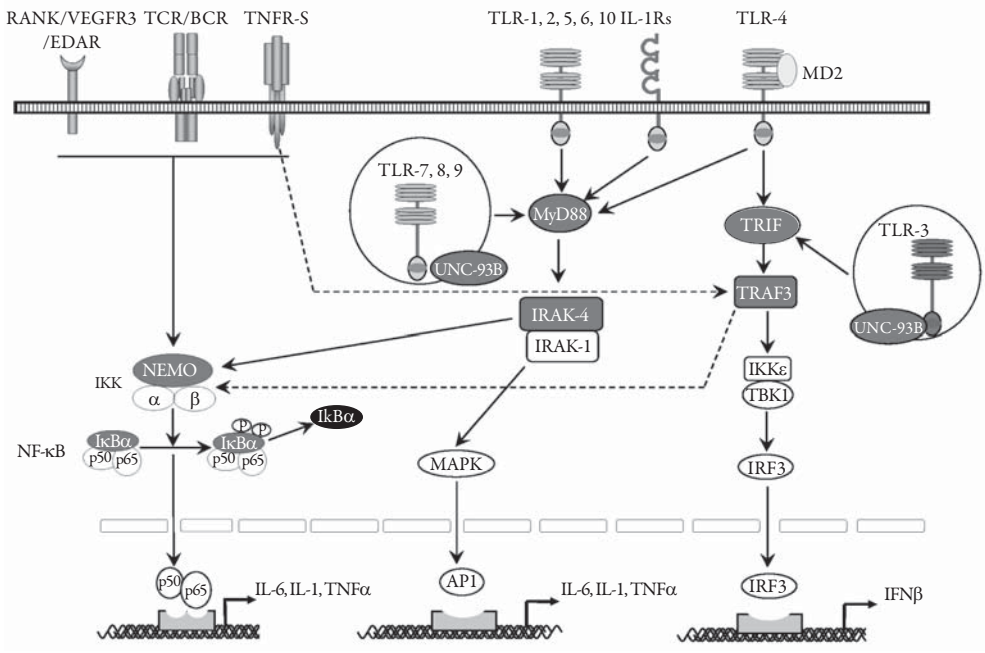

Figure 36.1 *Human defects involving NF-κB pathways.* Immune receptor signaling pathways leading to NF-κB activation can be grouped into four categories on the basis of the surface receptors involved: developmental receptors RANK, VEGFR3, and EDAR; antigen receptors (TCR and BCR); members of the TNF receptor superfamily (TNF-Rs); and members of the TIR superfamily (IL-1Rs/TLRs). The two proteins of the TIR signaling pathway (MyD88, IRAK-4) and the two proteins of the NF-κB signaling pathway (NEMO and IκBα) responsible for primary immunodeficiency are shown in gray. The defect in UNC-93B abolishes cellular responses to TLR3, TLR7, TLR8, and TLR9 agonists. The defect in TRAF3 impairs cellular responses to TLR3 and other pathways. The four proteins of the TLR3 signaling pathway (TLR3, UNC-93B, TRIF, and TRAF3) responsible for immunodeficiency selective for herpes simplex virus 1 encephalitis (HSE) are shown in gray.

critical amino-terminal serine residues by the IκB kinase (IKK) complex, leading to their ubiquitination and subsequent degradation (Fig. 36.1; Puel et al., 2004). The IKK complex consists of at least three related catalytic subunits, IKKα, IKKβ, and IKKγ/NEMO (for NF-κB essential modulator) (Israel, 2010). The degradation of IκBs results in the translocation of NF-κB dimers to the nucleus, where they bind to DNA at cognate binding sites and regulate gene transcription (Puel et al., 2004). The classical proinflammatory TIR signaling pathway leads to the synthesis of inflammatory cytokines and chemokines, such as IL-1β, -6, -8, and -12, and tumor necrosis factor α (TNF-α) (Fig. 36.1; Kawai and Akira, 2010). The alternative pathway is controlled by TRIF, which is used by TLR-3 and TLR-4, leading to activation of the transcription factors IRF3 and NF-κB (Fig. 36.1; Kawai and Akira, 2010). TRIF recruits TRAF6 and activates TAK1 for NF-κB activation. TRIF also recruits a signaling complex involving TBK1 and IKKε via TRAF3 for IRF3 activation (Kawai and Akira, 2010). This signaling pathway induces the production of type I and type III IFNs and inflammatory cytokines and is important in antiviral immunity (Kawai and Akira, 2010).

Four primary immunodeficiencies (PIDs) associated with impaired signaling via the TIR canonical pathway have been reported, with mutations in *IRAK4*, *MYD88*, *NEMO*, and *IKBA* (Fig. 36.1) (Courtois et al., 2003; Doffinger et al., 2001; Hanson et al., 2008; Picard et al., 2003, 2010; von Bernuth et al., 2008). The mutations in *NEMO* and *IKBA* also impair the alternative, TRIF-dependent pathway. The principal infectious phenotype of patients with any of these four defects is the occurrence of pyogenic bacterial infections. Conversely, four genetic defects affect primarily the alternative pathway, with mutations in *TLR3*, *UNC93B1*, *TRIF* and *TRAF3* (Fig. 36.1; Casrouge et al., 2006, Perez de Diego et al., 2010; Zhang et al., 2007) (Sancho-Shimizu et al.2011). *UNC93B1* and *TRAF3* defects also impair the TLR7-9 pathway, but with no known clinical consequences. The predominant infectious phenotype of patients with any of these three defects is herpes simplex virus 1 encephalitis (HSE) in childhood. Collectively, these seven disorders were initially thought to be rare, but they have since been diagnosed in about 200 patients around the world. They have very little in common except that they all impair at least one arm of NF-κB–involved immunity. We summarize here the infectious diseases seen in patients with mutations impairing the canonical pathway and the alternative pathway. We also discuss the diagnostic and therapeutic options in such patients, in an attempt to propose tentative guidelines for clinicians.

INBORN ERRORS OF THE TLR AND IL-1R CANONICAL PATHWAY (MYD88 AND IRAK-4)

HUMAN IRAK-4 DEFICIENCY

Molecular Basis of the Disease

Autosomal recessive IRAK-4 deficiency was discovered in 2003 (Picard et al., 2003). Up to 52 patients have since been identified,

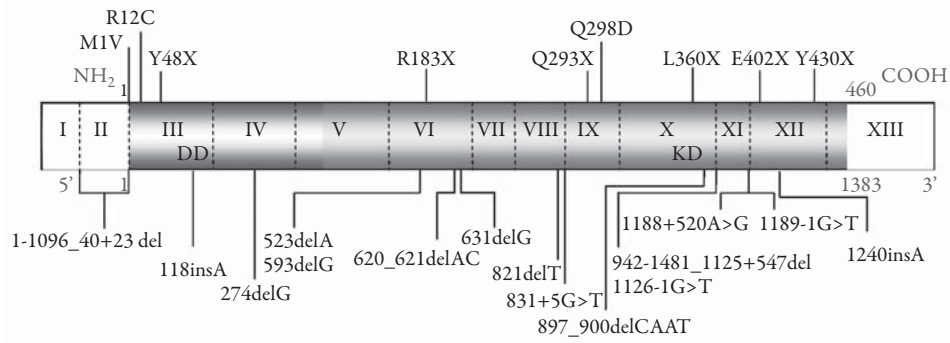

Figure 36.2 *Schematic diagram of the structure of the IRAK4 gene and the mutations identified.* Human *IRAK4* has 13 exons (Roman numerals), including two noncoding first exons, and encodes a protein (shown in gray) including a death domain (DD) and a kinase domain (KD). The position of all known mutations is represented (amino-acid nomenclature at the top, nucleotide nomenclature at the bottom).

from 33 kindreds, in 14 countries (Bouma et al., 2009; Cardenes et al., 2006; Chapel et al., 2005; Currie et al., 2004; Davidson et al., 2006; Day et al., 2004; Enders et al., 2004; Hoarau et al., 2007; Krause et al., 2009; Ku et al., 2007a, 2007b; Lavine et al., 2007; McDonald et al., 2006; Medvedev et al., 2003; Picard et al., 2010; Takada et al., 2006; van Bruggen et al., 2010; von Bernuth et al., 2006; Yang et al., 2005; and unpublished data). IRAK-4–deficient patients have homozygous or compound heterozygous mutations in the *IRAK4* gene (Fig. 36.2). Up to 17 cases have been identified as sporadic, with 35 cases considered familial (16 kindreds). The 33 affected families originated from 14 countries, from several different continents: the Americas (Canada, El Salvador, United States of America), Asia (Israel, Japan, Saudi Arabia, Turkey), Australia, and Europe (France, Hungary, Portugal, Slovenia, Spain, United Kingdom) (Fig. 36.3). Most of the patients and their families were living in their countries of origin, with the exception of a Portuguese family living in France, a Turkish family living in Germany, and a family from El Salvador living in the United States. The patients with IRAK-4 deficiency from 19 kindreds carried homozygous mutations, whereas those from 14 other kindreds were compound heterozygous for *IRAK4* mutations (Picard et al., 2010, and unpublished data). One patient was compound heterozygous for the Q293X mutation and for a large de novo deletion encompassing the *IRAK4* gene (Ku et al., 2007b). The mutations identified were nonsense mutations, missense mutations, splice mutations, and frameshift insertions or deletions (Fig. 36.2). All the mutations were loss of expression and loss of function, with the possible exception of two missense mutations, G298D and R12C, which seemed to be associated with residual IRAK-4 protein production (Picard et al., 2010).

Laboratory Findings and Immunological Features

In whole-blood assays, leukocytes from IRAK4-deficient patients do not produce IL-6, and CD62L shedding from

Figure 36.3 *Countries of origin of the 33 kindreds with IRAK-4 deficiency identified* (in dark gray). The number of patients identified in each country is indicated.

granulocytes does not occur in response to activation with the TLR and IL-1R agonists tested, with the exception of TLR3, which uses an IRAK-4–independent pathway (von Bernuth et al., 2006, 2008; Zhang et al., 2007). The defects observed abolished all TLR responses (with the exception of those to TLR3 and a few TLR4 responses), and all IL-1R responses (at least IL-1β, IL-18, and IL-33) tested, in all hematopoietic and nonhematopoietic cells from all patients tested (J. E. Sims, personal communication). However, there seems to be no overt defect of leukocyte development in IRAK-4–deficient patients and these patients have normal antigen-specific T- and B-cell responses, as shown by normal findings for immunological analyses, with three notable exceptions (Ku et al., 2007b; von Bernuth et al., 2008). First, the IgM+IgD+CD27+ cells but not switched B-cells were strongly reduced in IRAK-4-deficient patients (Weller el al. 2012). Second, the glycan-specific IgG and IgM antibody response to pneumococcal and AB glycans has been shown to be impaired in up to 50 percent of the patients explored (Picard et al., 2010). Third, serum IgE and IgG4 concentrations have been found to be high in 70 percent and 38 percent, respectively, of the patients tested (Picard et al., 2010). Nevertheless, none of the IRAK-4–deficient patients suffers from allergic asthma, and a chronic eczematous skin disease was reported in only one patient. IRAK-4 deficiency confers a predisposition to severe bacterial infection, with impairment of the ability to increase plasma C-reactive protein (CRP) concentrations and to mount fever at the beginning of infection. However, pus formation has been observed at various sites of infection. Finally, delayed separation of the umbilical cord is observed in 20 percent of IRAK-4–deficient patients.

Clinical and Pathological Manifestations

Despite their broad and profound immunological phenotype, patients with IRAK-4 deficiency display surprisingly narrow susceptibility to invasive pyogenic bacterial infections (meningitis, sepsis, arthritis, osteomyelitis, and abscesses). They also have normal resistance to common fungi, parasites, viruses and to many bacteria. Meningitis has been reported in 65 percent of IRAK-4–deficient patients, sepsis in 37 percent, arthritis in 27 percent, osteomyelitis in13 percent, and deep inner organ/tissue abscesses in 29 percent (Table 36.1). Only five IRAK-4–deficient patients have never developed invasive bacterial infection, and four of these patients were diagnosed at birth and have remained asymptomatic on prophylactic treatment. The invasive bacterial infections observed were mostly caused by *Streptococcus pneumoniae, Staphylococcus aureus*, and *Pseudomonas aeruginosa,* in particular. In IRAK-4–deficient patients, *S. pneumoniae* was involved in 54 percent of documented invasive episodes, whereas *S. aureus* and *P. aeruginosa* were found in 14 percent and 20 percent of such episodes, respectively (Table 36.2). Other gram-positive (*Streptococcus agalactiae, S. milleri, S. pyogenes*, and *S. parasanguis*) and gram-negative (*Shigella sonnei, Neisseria meningitidis*, *Haemophilus influenzae* type b, and *Clostridium septicum*) bacteria have been shown to cause invasive disease in IRAK-4–deficient patients

Table 36.1 PERCENTAGE OF IRAK-4- AND MYD88-DEFICIENT PATIENTS WITH INFECTIONS AT VARIOUS SITES

	TOTAL NUMBER OF PATIENTS (N = 74)	IRAK-4–DEFICIENT PATIENTS (N = 52)	MYD88-DEFICIENT PATIENTS (N = 22)
Meningitis	59%	65%	45%
Sepsis	41%	37%	50%
Arthritis	23%	27%	14%
Osteomyelitis	12%	13%	9%
Abscess	24%	29%	14%
Lymphadenitis	27%	29%	23%
Skin infection	35%	44%	14%
Pneumonia	19%	21%	14%
ENT	27%	33%	14%

Data from Conway et al., 2010; Picard et al., 2010; unpublished data.

(Table 36.2). Most IRAK-4–deficient patients (90 percent) suffered from their first bacterial infection before the age of 2 years. Twenty patients (38 percent) died from invasive bacterial infections, all before the age of 8 years and most before the age of 2 years (Picard et al., 2010; and unpublished data). Eleven of these patients died of invasive pneumococcal disease. However, this PID improved with age, and patients with IRAK-4 deficiencies do not seem to be particularly susceptible to invasive bacterial infection after their teens (Picard et al., 2010).

IRAK-4–deficient patients present also with noninvasive pyogenic bacterial infections, mostly of the skin and upper respiratory tract. Necrotizing infections are particularly common at these two sites. Recurrent localized skin infections (furunculosis, folliculitis, cellulitis, omphalitis, and orbital cellulitis or endophthalmitis) have been found in 44 percent of patients, lymphadenitis in 29 percent, and ear, nose, and throat (ENT) infections (otitis, sinusitis, tonsillar abscesses, necrotizing epiglottitis, pharyngitis, and palate infection) in 33 percent of patients (Table 36.1). Intriguingly, only 21 percent of patients have had pneumonia, and none has developed chronic bronchopulmonary disease. Of note, a pair of twins with this deficiency developed pneumonia during primary infection with cytomegalovirus (CMV) at the age of 3 months (unpublished data). The principal bacterial strains found during noninvasive infections were *S. aureus* in 43 percent of episodes, *P. aeruginosa* in 22 percent of episodes, and *S. pneumonia* in 16 percent of episodes in IRAK-4–deficient patients (Table 36.2). Other gram-positive (*S. pyogenes, S. intermedius,* β-hemolytic streptococci, and *S. equi*) and gram-negative (*E. coli, Serratia marcescens, M. catarrhalis*, and *Citrobacter freundii*) bacteria have also been reported to cause noninvasive disease in IRAK-4–deficient patients. Only one IRAK-4–deficient patient developed otitis and pneumonia due to *Mycobacterium avium*. All IRAK-4–deficient patients had noninvasive bacterial infections, and more than half the

Table 36.2 DOCUMENTED BACTERIAL INFECTION IN IRAK-4- AND MYD88-DEFICIENT PATIENTS

(PATIENTS N = 74)	INVASIVE INFECTION		NONINVASIVE INFECTION	
(N = DOCUMENTED INFECTION)	IRAK-4 (N = 106)	MYD88 (N = 44)	IRAK-4 (N = 63)	MYD88 (N = 15)
S. pneumoniae	54%	41%	16%	20%
S. aureus	14%	20%	43%	53%
Streptococcus spp.	6%	11%	8%	
S. milleri	1%			
S. pyogenes	3%		3%	
S. A group		2%		
S. β-*hemolytic*		5%	2%	
S. parasanguis	1%			
S. agalactiae	1%			
S. B group		2%		
S. intermedius			2%	
S. equi			2%	
P. aeruginosa	20%	16%	22%	13%
Gram-negative bacteria	7%	11%	10%	13%
Shigella sonnei	2%			
Neisseria meningitidis	2%			
Haemophilus influenzae	2%	2%		
Salmonella enteriditis		7%		
Klebsiella pneumoniae				7%
Escherichia coli			5%	7%
Serratia marcescens			2%	
Moraxella catarrhalis		2%	2%	
Clostridium septicum	1%			
Citrobacter freundii			2%	
Mycobaterium avium			2%	

Data from Conway et al., 2010; Picard et al., 2010; unpublished data.Picard

patients suffered from their first noninvasive bacterial infection before the age of 2 years. All patients continued to suffer from skin infections, sinusitis, or pneumonia, including those who had reached adulthood.

HUMAN MYD88 DEFICIENCY

Molecular Basis of the Disease

Autosomal recessive MyD88 deficiency was discovered in 2008 (von Bernuth et al., 2008). Up to 22 patients have since been identified, from seven kindreds in six countries (Conway et al., 2010; Picard et al., 2010; and unpublished data). MyD88-deficient patients have homozygous or compound heterozygous mutations in the *MYD88* gene (Fig. 36.4). Only 1 case has been described as sporadic, with 21 cases described as familial (six kindreds). The seven families originated from six countries in the Americas (United States of America), Asia (Turkey), and Europe (France, Portugal, Serbia, Spain). All patients and their families were living in their countries of origin, with the exception of a Serbian family living in Switzerland (Fig. 36.5). The patients of six kindreds with MyD88 deficiency were homozygous, and one patient was compound heterozygous (Picard et al., 2010; von Bernuth et al., 2008). The mutations identified in patients were one nonsense mutation, two missense mutations, and one frameshift deletion (Fig. 36.4). All the mutations identified are loss of function, but only one *MYD88* mutation (E66X) is loss of expression (Conway et al., 2010). Two mutant *MYD88* alleles have been shown to lead to the production of very small amounts of a nonfunctional protein (E65del and L106P), whereas the R209C mutant allele is associated with quantitatively normal production of a nonfunctional protein (Picard et al., 2010; von Bernuth et al., 2008).

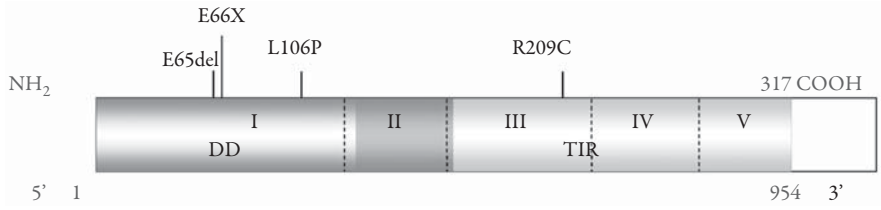

Figure 36.4 *Schematic diagram of the structure of the MYD88 gene and the mutations identified.* Human *MYD88* has five exons (Roman numerals) encoding a protein (shown in gray) with TIR and DD domains. The position of all known mutations is represented in amino-acid nomenclature at the top.

Laboratory Findings and Immunological Features

MyD88-deficient patients display a lack of production of IL-6 by whole blood, and CD62L shedding from granulocytes is absent following activation with most of the TLR and IL-1R agonists tested, with the exception of TLR3, which uses a MyD88-independent pathway (von Bernuth et al., 2006, 2008; Zhang et al., 2007). Thus, there seems to be no overt defect of leukocyte development in MyD88-deficient patients, and antigen-specific T- and B-cell responses appear to be normal in most cases, as shown by routine immunological analyses with three notable exceptions (Ku et al., 2007b; von Bernuth et al., 2008). First, the IgM+IgD+CD27+ cells but not switched B-cells were strongly reduced in MyD88-deficient patients (Weller el al. 2012). Second, serum IgE and IgG4 concentrations were high in up to half and in 28 percent, respectively, of the patients tested (Picard et al., 2010). Third, some of the modest subclinical abnormalities of B-cell responses, such as the production of low levels of antibodies against carbohydrates in some patients, may thus reflect impaired TACI responses rather than impaired TLR and IL-1R responses. Indeed, MyD88 has recently been shown to control signaling downstream of TACI (He et al., 2010). MyD88 deficiency confers a predisposition to severe bacterial infection, with impairment of the ability to increase plasma CRP concentrations and to mount fever at the beginning of infection. However, pus formation has been observed at various sites of infection.

Clinical and Pathological Manifestations

Patients with MyD88 deficiency present a narrow susceptibility to invasive pyogenic bacterial infections (meningitis, sepsis, arthritis, osteomyelitis, and abscesses) and have normal resistance to common fungi, parasites, viruses and many bacteria. Meningitis has been found in 45 percent of MyD88-deficient patients, sepsis in 50 percent, arthritis in 14 percent, osteomyelitis in 9 percent, and deep inner organ/tissue abscesses in 14 percent (Table 36.1) (Conway et al., 2010; Picard et al., 2010; and unpublished data). Only two MyD88-

Figure 36.5 *Countries of origin of the seven kindreds with MyD88 deficiency identified* (in light gray). The number of patients identified in each country is indicated.

deficient patients have never developed invasive bacterial infection (Conway et al., 2010). In MyD88-deficient patients, *S. pneumoniae* was involved in 41 percent of the documented invasive episodes, whereas *S. aureus* and *P. aeruginosa* were found in 20 percent and 16 percent of such episodes, respectively (Table 36.2). Other gram-positive bacteria (group A, B, and β-hemolytic streptococci) and gram-negative (*S. enteritidis, H. influenzae* type e, and *M. catarrhalis*) have been found to cause invasive disease in MyD88-deficient patients (Table 36.2) (Conway et al., 2010; Picard et al., 2010; and unpublished data). Most MyD88-deficient patients suffered from their first bacterial infection before the age of 2 years. Nine patients (41 percent) died from invasive bacterial infections, all before the age of 4 years and most before the age of 1 year (Conway et al., 2010; Picard et al., 2010; and unpublished data). Seven of these patients died from invasive pneumococcal disease (Conway et al., 2010; Picard et al., 2010; and unpublished data). However, MyD88 deficiencies seem to improve with age, and none of the patients has presented with invasive bacterial infection after adolescence (Picard et al., 2010).

MyD88 deficiencies also confer a predisposition to noninvasive pyogenic bacterial infections, mostly affecting the skin and upper respiratory tract. Necrotizing infections are particularly common at these two sites. Recurrent localized skin infections (furunculosis, folliculitis, cellulitis, and endophthalmitis) have been found in 14 percent of patients, lymphadenitis in 23 percent, and ENT infections (otitis, sinusitis, tonsillar abscesses, necrotizing epiglottitis, pharyngitis) in 14 percent of patients (Table 36.1) (Conway et al., 2010; Picard et al., 2010; and unpublished data). Only 14 percent of the patients had pneumonia, and none developed chronic bronchopulmonary disease. The principal bacterial strains found during noninvasive infections were *S. aureus* in 53 percent of patients, *S. pneumoniae* in 20 percent of patients, and *P. aeruginosa* in 13 percent of patients (Table 36.2). Other gram-negative bacteria (*E. coli* and *K. pneumoniae*) have been found to cause noninvasive disease in MyD88-deficient patients (Table 36.2). Severe mycobacterial, viral, parasitic, or fungal diseases have not been reported. Most MyD88-deficient patients have had noninvasive bacterial infections, with half the patients suffering from their first noninvasive bacterial infection before the age of 2 years. MyD88-deficient patients continue to suffer from skin infections, sinusitis, or pneumonia, including those who have reached adolescence.

TREATMENT OF IRAK-4 AND MYD88 DEFICIENCIES

MyD88 and IRAK-4 deficiencies are phenocopies in terms of their immunological and clinical phenotypes (von Bernuth et al., 2008), so the management of these two deficiencies can be discussed together. Patients with IRAK-4 and MyD88 deficiencies should be immunized with conjugated and nonconjugated *S. pneumoniae* vaccines, conjugated *H. influenzae* vaccine, and conjugated and nonconjugated *N. meningitidis* vaccines. A preventive treatment regimen, including antibiotic prophylaxis with cotrimoxazole plus penicillin V (or equivalent), is required throughout the patient's life, with empirical intravenous (IV) or subcutaneous (SC) immunoglobulin injections until at least the age of 10 years. This prophylaxis seems to have an impact on the incidence of invasive bacterial infections (Picard et al., 2010). Both clinical status and outcome improve with age, and prophylactic treatment appears to be beneficial in these patients. This dramatic improvement with age may be accounted for by the development of adaptive antigen-specific T- and B-lymphocyte responses.

INBORN ERRORS OF THE CORE NF-κB–MEDIATED SIGNALING PATHWAY

HUMAN NEMO DEFICIENCY

Molecular Basis of the Disease

X-linked recessive (XR) anhidrotic ectodermal dysplasia (EDA) with immunodeficiency (ID) due to hypomorphic *IKBKG/NEMO* mutations was described in 2000 (Zonana et al., 2000) and in 2001 (Doffinger et al., 2001) as a disorder caused by an impairment of NF-κB immunity. NEMO is a regulatory subunit of the IKK complex (Puel et al., 2004). Approximately 100 male patients with hemizygous hypomorphic mutations of *NEMO* have been reported to date, and about 47 different mutations have been identified. All mutations functionally investigated have been found to result in at least some impairment of NF-κB activation (Aradhya et al., 2001; Chang et al., 2008; Dai et al., 2004; Devora et al., 2010; Doffinger et al., 2001; Dupuis-Girod et al., 2002; Filipe-Santos et al., 2006; Fusco et al., 2008; Hanson et al., 2008; Haverkamp et al., 2004; Jain et al., 2001; Ku et al., 2005, 2007a; Lee et al., 2005; Martinez-Pomar et al., 2005; Mooster et al., 2010; Niehues et al., 2004; Orange et al., 2002, 2004a, 2004b, 2005; Orstavik et al., 2006; Pachlopnik Schmid et al., 2006; Puel et al., 2006; Roberts et al., 2010; Salt et al., 2008; Smahi et al., 2000; Zonana et al., 2000, and unpublished data). Two female patients with defective X chromosome lyonization have also been reported (Kosaki et al., 2001; Martinez-Pomar et al., 2005). Patients with this deficiency have been identified in 14 countries from various continents, including Africa (South Africa), the Americas (Canada, United States of America), Asia (Japan, Turkey), and Europe (Belgium, France, Germany, Italy, Poland, Netherlands, Norway, Switzerland, United Kingdom), including missense, nonsense, splice-site mutations, and frameshift insertions and deletions (Figs. 36.6 and 36.7). NF-κB dimers are involved in several pathways, including those triggered by the many members of the TNF-R, IL-1R, T-cell receptor (TCR), B-cell receptor (BCR), and TLR families (Fig. 36.1). In cases of NEMO deficiency, the impairment of the various immunological pathways depends on the mutation concerned (type and location), with between one and all the pathways impaired (Filipe-Santos et al., 2006; Hanson et al., 2008; Mooster et al., 2010; Puel et al., 2004). There is considerable allelic heterogeneity, accounting for the tremendous variability of cellular and clinical phenotypes.

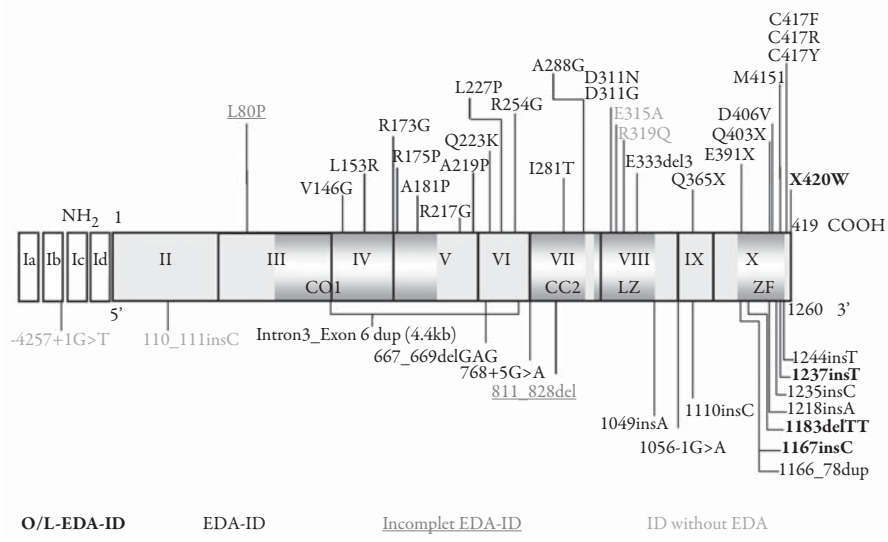

Figure 36.6 *Schematic diagram of the structure of the NEMO gene and the mutations identified.* Human *NEMO* has 10 exons (Roman numerals), including four noncoding alternative exons. *NEMO* encodes a protein (shown in gray) with two coil-coiled (CC) domains, one leucine zipper (LZ) and one zinc-finger (ZF) domain. The position of all mutations reported is represented (amino-acid nomenclature at the top, nucleotide nomenclature at the bottom). Mutations responsible for the osteopetrosis/lymphedema-EDA-ID phenotype are shown in black bold, mutations responsible for the EDA-ID phenotype are shown in black, mutations responsible for an incomplete EDA-ID phenotype (conical teeth only) are shown in light gray underline, and mutations responsible for ID without an EDA phenotype are shown in light gray.

Laboratory Findings and Immunological Features

NEMO-deficient patients generally display a lack of IL-10 production in response to activation with TNF-α in whole-blood assays (Doffinger et al., 2001; Hanson et al., 2008; Puel et al., 2004). TLR and IL-1R signaling pathways have been explored in only a few patients and have been shown to be impaired in patients with the most severe clinical infectious phenotype, with a high degree of susceptibility to infection (Hanson et al., 2008). However, they may be intact in certain individuals. Almost all patients bearing mutations in *NEMO* have an impaired antibody response, to glycans, including pneumococcal capsules, in particular (Hanson et al., 2008; Puel et al., 2004). Half the patients also have hypogammaglobulinemia, presumably

Figure 36.7 *Countries of origin of the patients with NEMO deficiency identified* (in gray).

secondary to a CD40 signaling impairment found in most patients with mutations affecting the zinc-finger domain (Hanson et al., 2008). High serum IgM concentrations have been found in 15 percent of patients with hyper-IgM–like phenotype, and high levels of IgA have been found in 37 percent of patients (Hanson et al., 2008). More than 10 patients have been explored for natural killer (NK) function, and all were found to have impaired NK-cell cytotoxicity, despite having normal numbers of peripheral blood NK cells (Hanson et al., 2008; Orange et al., 2002; and unpublished data). Other immunological parameters used for diagnosis, such as the numbers of blood phagocytes, B and T lymphocytes, lymphocyte subset distribution (CD4, CD8), and T-cell proliferation in response to mitogens and antigens, are generally normal in most patients tested (Hanson et al., 2008). However, one NEMO-deficient patient with somatic reversion mosaicism in T, B, and NK cells revealed the critical role of NEMO in lymphocyte development and/or survival (Nishikomori et al., 2004). The severity and diversity of the infectious phenotype of NEMO-deficient patients and the diversity of signaling pathways potentially affected by mutations in the corresponding gene thus contrast with the small number of detectable abnormalities identified in routine immunological examination of affected patients.

Clinical and Pathological Signs

The broad and profound immunological phenotypes of patients with NEMO deficiencies are responsible for a high degree of susceptibility to invasive pyogenic bacterial infections (meningitis, sepsis, arthritis, osteomyelitis, and deep organ abscesses), environmental mycobacteria, parasites, viruses, and fungal infections. One third of the patients have had sepsis, one third have had a deep tissue abscess, one third have had recurrent pneumonia with bronchiectasis, 20 percent have had meningitis or encephalitis, about 23 percent have had gut infection, and 11 percent have had osteomyelitis and sinusitis (Table 36.3) (Doffinger et al., 2001; Hanson et al., 2008; Puel et al., 2004). Pyogenic bacterial infection has been identified in about 86 percent of NEMO-deficient patients and has been attributed to several species, including *S. pneumoniae*, *H. influenzae*, and *S. aureus*. Mycobacterial infection is found in about 44 percent of NEMO-deficient patients (cellulitis, osteomyelitis, lymphadenitis, pneumonia, and disseminated infections) and is mostly caused by *M. avium* or *M. kansasii* (Doffinger et al., 2001; Hanson et al., 2008). The presence of mycobacterial infection in patients with NEMO deficiency is indicative of a particularly poor prognosis. Serious viral infection has occurred in 21 percent of NEMO-deficient patients (herpes simplex virus encephalitis, severe adenoviral gastroenteritis, severe cytomegalovirus infection) (Table 36.4) (Doffinger et al., 2001; Hanson et al., 2008; Niehues et al., 2004; Puel et al., 2006). Finally, opportunistic infections, *Pneumocystis jirovicii* infections, and chronic mucocutaneous candidiasis (CMC) have been reported, but in less than 10 percent of patients (Doffinger et al., 2001; Hanson et al., 2008; Orstavik et al., 2006). However, the number of *Pneumocystis* infections recognized as associated with NEMO deficiency and even as a presenting infection has increased in recent years (Hanson et al., 2008; Salt et al., 2008). In summary, the spectrum of infectious diseases is broad in NEMO-deficient patients, as most patients present with multiple infections (Bustamante et al., 2008). Almost all patients report infections caused by pyogenic bacteria (*S. pneumoniae*, *S. aureus*, *P. aeruginosa*, and

Table 36.3 PERCENTAGE OF PATIENTS WITH NEMO- AND *IKBA* MUTATIONS WITH INFECTIONS AT VARIOUS SITES

	NEMO (ADAPTED FROM HANSON ET AL., 2008)	IKBA (N = 6 PATIENTS)
Meningitis/encephalitis	20%	14% (1/7)
Sepsis	33%	29% (2/7)
Arthritis/osteomyelitis	11%	14% (1/7)
Abscess	30%	14% (1/7)
Gut infection/diarrhea	23%	86% (6/7)
Pneumonia	31%	86% (6/7)
ENT	11%	29% (2/7)

Data from Dupuis-Girod et al., 2006; Hanson et al., 2008; Janssen et al., 2004, Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et al. 2012; E. Renner and T. Torgerson, personal communication.

Table 36.4 DOCUMENTED INFECTION IN PATIENTS WITH NEMO- AND *IKBA* MUTATIONS

	INFECTION	
	NEMO (ADAPTED FROM HANSON ET AL., 2008)	IKBA (N = 7 PATIENTS)
Bacterial infection	86%	7 pts
S. pneumoniae	>10%	-
S. aureus	>10%	1 pt
Streptococcus (S. A group)		1 pt
P. aeruginosa	>10%	1 pt
Haemophilus influenzae	>10%	-
Salmonella typhimurium		1 pt
Klebsiella pneumoniae		1 pt
Serratia marcescens		1 pt
Environmental mycobacteria	44%	-
Fungal infections		5 pts
C. albicans	10%	5 pts
P. jirovicii	8%	3 pts
Severe viral infections (HSV, CMV, or adenovirus)	21%	1 pt

Data from Dupuis-Girod et al., 2006; Hanson et al., 2008; Janssen et al., 2004, Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et la. 2012; E. Renner and T. Torgerson, personal communication.

Table 36.5 PHENOTYPE FREQUENCY OF SHARED MUTATIONS

MUTATION	768+5 G>A	L227P	E315A	R319Q	1167INSC	E391X	1218INSA	C417R	C417F	X420W
Patient number	12	3	4	2	6	3	2	7	2	2
Alive	0%	33%	67%	100%	33%	100%	0%	50%	100%	0%
EDA	100%	100%	25%	0%	100%	100%	100%	100%	100%	100%
Pyogenic bacterial infection	80%	100%	50%	50%	83%	100%		100%	100%	100%
Severe viral infection	0%	0%	0%	0%	50%	0%		0%	50%	50%
Mycobacterial infection	20%	0%	100%	100%	67%	0%		33%	0%	100%
Inflammatory bowel disease	71%	33%				100%				50%
Hypogammaglobulinemia	75%	100%	0%	0%	75%	0%		100%	100%	100%
Hyper-IgM	0%	0%	0%	0%	0%	0%		100%	100%	0%
Hyper-IgA	33%	0%	0%	0%	0%	100%		33%	100%	0%
Specific antibody defect	100%	100%	0%	50%	33%	50%		100%	100%	100%
TLR signaling defect	100%		0%	0%	100%			0%		100%

Adapted from Hanson et al., 2008.

H. influenzae), and only some patients suffer from opportunistic infectious diseases caused by mycobacterial, fungal, and/or viral infections.

About 80 percent of the NEMO-deficient patients reported to date have had isolated EDA characterized by the presence of sparse hair, dental abnormalities (conical teeth, tooth agenesis), and hypohidrosis with lack of sweating and a tendency to develop hyperpyrexia (Hanson et al., 2008; Puel et al., 2004). These features result from defective signaling via the ectodysplasin receptor signaling pathway, the normal functioning of which is required for normal ectodermal development. Unrelated mutations in the genes encoding the ectodysplasin receptor or its ligand result in other forms of EDA, not associated with immunodeficiency. In some other NEMO-deficient patients, osteopetrosis and lymphedema have been found associated with the EDA phenotype (Doffinger et al., 2001; Hanson et al., 2008; Roberts et al., 2010) This is particularly true for those associated with a frameshift run-on mutation resulting in elongation of the NEMO protein. Some patients also have dysmorphia with mild frontal bossing (Dupuis-Girod et al., 2002; Mancini et al., 2008; and unpublished data). About 10 percent of NEMO-deficient patients have none of the features of the EDA phenotype (Hanson et al., 2008; Mooster et al., 2010; Puel et al., 2006). Inflammatory conditions are also frequent, with cutaneous inflammation and colitis. Some patients develop a disseminated skin eruption, dermatitis, eczema, and/or erythema early in life (Fish et al., 2009; Mancini et al., 2008; Minakawa et al., 2009; Permaul et al., 2009; Roberts et al., 2010; Tono et al., 2007; and unpublished data). Histological studies of the skin have revealed acanthosis, spongiosis of the epidermis, and a perivascular lymphohistiocytic infiltrate with necrotic keratinocytes (Mancini et al., 2008; Tono et al., 2007). Colitis was also found in 21 percent of patients, and some had intractable diarrhea with failure to thrive (Hanson et al., 2008; Orange et al., 2002, 2004a; Pai et al., 2008; Roberts et al., 2010; and unpublished data). There seems to be some evidence for a genotype–phenotype correlation in NEMO deficiency. Given the large number of mutations reported to date, it is difficult to be certain that some of the findings attributed to particular genotypes are relatively consistent. For example, mutations affecting specific regions of the NEMO protein tend to be associated with particular types of infection, the absence of an EDA phenotype, autoinflammation, osteopetrosis/lymphedema, and certain immune phenotypes, such as antibody defects and TLR impairments. A summary of the most frequently identified genotypes and their associated phenotypes is provided in Table 36.5. In conclusion, the developmental and immunological phenotype of NEMO-deficient patients is variable and probably relates to the biological function of each mutation.

HUMAN MUTATIONS IN *IKBA* GENE

Molecular Basis of the Disease

In 2003, an autosomal dominant (AD) form of EDA-ID was identified that was caused by a hypermorphic heterozygous mutation of *NFKBIA/IKBA*, impairing the phosphorylation and degradation of NF-κB inhibitor α (IκBα) (Fig. 36.1) (Courtois et al., 2003). Seven patients with four different hypermorphic mutations of *NFKBIA/IKBA* have since been identified (Fig. 36.8) (Courtois et al., 2003; Janssen et al., 2004; Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et al. 2012; and E. Renner and T. Torgerson, personal communication). The patients originated from five countries: North America (United States of America), Asia (Japan) and Europe (Italy, Netherlands, Germany). Two of the mutations identified were missense mutations and three were nonsense mutations (Fig. 36.8). The same heterozygous 94G>T mutation was identified in two unrelated kindreds. This mutation is responsible for the replacement of a serine residue important for the phosphorylation of IκBα with an isoleucine residue (S32I). The S32I,

Q9X and Q14X mutations are gain-of-function mutations, as they increase the inhibitory capacity of IκBα by preventing its phosphorylation and degradation, resulting in the impairment of NF-κB activation (Courtois et al., 2003; Janssen et al., 2004; Lopez-Granados et al., 2008; and Ohnishi et al. 2012). The W11X nonsense mutation is responsible for haploinsufficiency and less severe clinical, immunological, and functional phenotypes in the patient bearing this mutation (McDonald et al., 2007). Functional studies are currently under way for the M37K mutation (E. Renner and T. Torgerson, personal communication). One IκBα-mutated adult patient with a S32I mutation presented a complex partial mosaicism; his son also presents the same mutation of *IKBA*, but with no mosaicism (Janssen et al., 2004). IκB molecules are involved in several pathways, including those triggered by the many members of the TNF-R, IL-1R, TCR, BCR, and TLR families. IκBα mutations leads to a particularly severe impairment of TCR signaling.

Laboratory Findings and Immunological Features

The three children bearing AD mutations (two patients bearing S32I and one bearing W37K) in the *IKBA* gene without mosaicism had hypogammaglobulinemia with hyper-IgM and no production of specific antibodies (Courtois et al., 2003; Janssen et al., 2004; and E. Renner, personal communication). These three patients and the pediatric patient with the Q14X mutation had low proportions of memory CD4 and CD8 T cells and no TCR gamma/delta T cells. Five patients displayed severe impairment of T-cell proliferation in response to anti-CD3 or PHA, but antigen-mediated T-cell proliferation was impaired only in the two patients carrying the S32I mutation without mosaicism (Courtois et al., 2003; Janssen et al., 2004; Lopez-Granados et al., 2008; Ohnishi et al. 2012; and E. Renner and T. Torgerson, personal communication). The child with the W11X mutation presented only a defect of glycan antibody production (McDonald et al., 2007). No immunological abnormalities were found in the adult patient with complex mosaicism and the S32I mutation, but the production of specific glycan antibodies was not explored in this patient (Janssen et al., 2004).

Clinical and Pathological Signs

The broad and profound immunological phenotypes of patients with *IKBA* mutations are responsible for broad susceptibility to severe pyogenic bacterial infections (meningitis, sepsis, arthritis, enteritis, abscesses, and pneumonia) and fungi. All seven patients have developed recurrent pyogenic bacterial infections. All presented with recurrent bacterial pneumonia, three have had sepsis or meningitis, and one has had arthritis (Table 36.3) (Dupuis-Girod et al., 2006; Janssen et al., 2004; Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et al. 2012; and E. Renner and T. Torgerson, personal communication). Pyogenic bacterial infections were identified in all IκBα-deficient patients, due to β-hemolytic type A streptococci, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *S. marcescens*, and *S. typhimurium*. Patients are also prone to opportunistic infections, with three having had *P. jirovicii* infections. Four patients has had CMC and one has had hepatitis caused by CMV (Table 36.4). Finally, six of these patients presented with recurrent diarrhea and/or colitis. The patient with the W11X mutation responsible for haploinsufficiency and the adult patient with complex partial mosaicism and the S32I mutation did not develop opportunistic infections, chronic diarrhea, or failure to thrive (Janssen et al., 2004; McDonald et al., 2007). All IκBα-deficient patients had EDA, with sparse hair, abnormal teeth (conical teeth, tooth agenesis), and hypohidrosis (Dupuis-Girod et al., 2006; Janssen et al., 2004, Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et al. 2012; E. Renner and T. Torgerson, personal communication), with the exception of the adult patient with complex partial mosaicism, who did not display features of EDA (Janssen et al., 2004).

TREATMENT AND PROGNOSIS OF PATIENTS WITH *IKBA* AND *NEMO* MUTATIONS

Prophylactic antibiotic treatment with cotrimoxazole and penicillin V (or equivalent) for preventing pyogenic bacterial infection, and with azithromycin (or equivalent) to prevent atypical mycobacterial infection, should be offered to all patients with mutations in *IKBA* and *NEMO* genes. IV or SC immunoglobulin substitution should also be considered, given the frequency and range of impaired B-cell immunity. Patients with mutations in *IKBA* and *NEMO* genes with functional B-cell immunity can be immunized with conjugated and nonconjugated *S. pneumoniae* vaccines, conjugated *H. influenzae* vaccine, and conjugated and nonconjugated *N. meningitidis* vaccines, irrespective of the provision of Ig therapy. Hematopoietic stem-cell transplantation (HSCT) has been reported in two children mutated

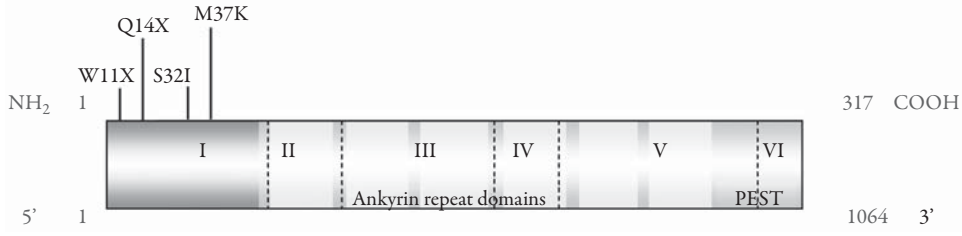

Figure 36.8 *Schematic diagram of the structure of the IKBA gene and the mutations identified.* Human *IKBA* has six exons (Roman numerals) encoding a protein (shown in gray) with ankyrin repeat domains (light gray) and PEST (proline/glutamic acid/serine/threonine) domains. The position of all mutations reported is represented in amino-acid nomenclature at the top.

in *IKBA* gene with combined immunodeficiency (Dupuis-Girod et al., 2006; Fish et al., 2009). One of these patients is alive and well in the absence of treatment, 8 years after parental haploidentical HSCT, whereas the second patient died of bacterial sepsis during the aplasia period (Dupuis-Girod et al., 2006; Fish et al., 2009). Seven NEMO-deficient patients with severe clinical and immunological phenotypes have undergone transplantation with various conditioning regimens (ranging from myeloablative to reduced-intensity conditioning) and with a matched related donor or a partially matched unrelated donor (Dupuis-Girod et al., 2006; Fish et al., 2009; Minakawa et al., 2009; Pai et al., 2008; Permaul et al., 2009; Tono et al., 2007; and unpublished data). Two patients died after HSCT, one from veno-occlusive disease and the other from *Parainfluenza* type III viral infection (Dupuis-Girod et al., 2002; Fish et al., 2009). Five NEMO-deficient patients displayed engraftment and correction of their immunodeficiency, but the preexisting colitis in two of these patients was either exacerbated or not cured (Fish et al., 2009; Pai et al., 2008; Permaul et al., 2009; Tono et al., 2007; and unpublished data). HSCT can correct these immunodeficiencies, but some inflammatory signs may persist or even become worse and the EDA phenotype is not corrected. This difficult procedure should be proposed only for selected patients with severe immunodeficiency, with the most closely matched donor available. A large international clinical survey of NEMO-deficient patients is currently under way to improve definition of the clinical and immunological outcome of these patients (Picard and Orange, in progress). This study may facilitate the formulation of guidelines for the treatment of this heterogeneous genetic disorder.

INBORN ERRORS OF THE TLR3-IFN-A, IFN-B, AND IFN-Λ PATHWAY

HUMAN TLR3 DEFICIENCY

Molecular Basis and Laboratory Findings

In 2007, an autosomal dominant form of TLR3 deficiency (Fig. 36.1) due to a heterozygous mutation of *TLR3* with dominant-negative effect was identified (Zhang et al., 2007). Seven patients from two unrelated kindreds with the same missense P554S mutation of *TLR3* have been reported, (Fig. 36.9). Both kindreds originate from France. Two of them had developed herpes simplex encephalitis (HSE) during childhood. Blood leukocytes (monocyte-derived dendritic, NK, and CD8 T cells) from TLR3-deficient patients have an impaired response to stimulation with TLR3 agonist. The fibroblast cells of the patients produce low levels of the antiviral molecules IFN-β and IFN-λ in response to TLR3 agonist, herpes simplex virus 1 (HSV-1), and vesicular stomatitis virus (VSV), leading to higher levels of viral replication and virus-induced cell death than for healthy control cells. All standard immunological parameters, such as the numbers of blood phagocytes, B and T lymphocytes, lymphocyte subset distribution (CD4 T, CD8 T), NK cells, antibody production, and T-cell proliferation in response to the mitogens and antigens tested, were normal in all patients tested. These observations suggest that the TLR3-dependent generation of IFN-α, IFN-β, and IFN- λ is critical for primary immunity to HSV-1 in the central nervous system (CNS) but redundant for immunity to most other viral infections (Zhang et al., 2007).

The recent report of an autosomal recessive form of complete TLR3 deficiency in a young adult who had developed HSE during childhood further illustrates the crucial importance of TLR3 signaling in fibroblasts for protective immunity to HSV-1 in the CNS (Guo et al. 2011). While the TLR3 activation in leukocytes from the TLR3-deficient patient resulted in normal antiviral IFN production.

Clinical and Pathological Manifestations and Treatment

Only one child from each of the two kindreds with autosomal dominant TLR3 deficiency (two of the seven individuals bearing the TLR3 mutation) developed HSE. The first patient developed HSE at 5 years of age, during primary infection with HSV-1. HSE was diagnosed on the basis of the intrathecal synthesis of specific anti–HSV-1 antibodies. The patient was treated with IV acyclovir and recovered well. Nineteen months later, HSE recurred, with the detection of HSV-1 nucleic acid in cerebrospinal fluid (CSF), associated with recent left temporal lesions detected by cerebral imaging. The patient was treated again with acyclovir, IV at first and then orally. The second patient presented an episode of meningoencephalitis at the age of 5 months, diagnosed

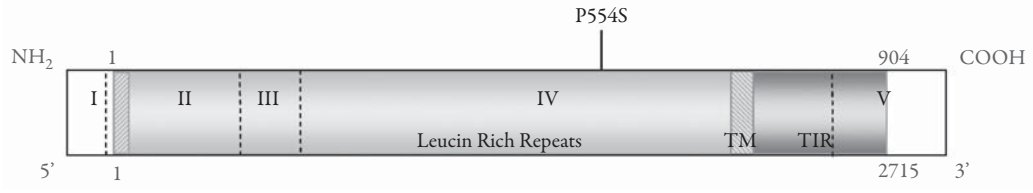

Figure 36.9 *Schematic diagram of the structure of the TLR3 gene and the mutation identified.* Human TLR3 has five exons (Roman numerals) encoding a protein (shown in gray) with a leucine-rich repeat (LRR) domain, a transmembrane (TM) domain, and a Toll/interleukin-1 receptor (TIR) domain. The position of the mutation reported is represented with amino-acid nomenclature.

following the detection of HSV-1 antigen and IFN-α in the CSF sample. The patient was treated with IV acyclovir and recovered well, with no recurrence. Both patients have been exposed to other viruses during 8 and 20 years of follow-up, respectively, as shown by the positive serological results obtained for other herpes viruses, including varicella zoster virus (VZV) and Epstein-Barr virus (EBV), with no subsequent acute events. The patients were also immunized with live mumps/rubella/measles and poliovirus vaccines, with no adverse effect. They did not experience infections caused by agents other than viruses, such as pyogenic bacteria or mycobacterial, parasitic, and fungal agents. None of the other five individuals with the dominant *TLR3* mutation developed HSE, despite serologically documented HSV-1 infection. TLR3 deficiency thus displayed complete penetrance at the cellular level but incomplete penetrance at the clinical level. Multiple factors may affect clinical penetrance, including age at infection with HSV-1, the viral inoculum, and human modifier genes.

HUMAN UNC-93B DEFICIENCY

Molecular Basis and Laboratory Findings

In 2006, autosomal recessive UNC-93B deficiency was identified as the first genetic etiology of isolated HSE (Casrouge et al., 2006). UNC-93B is a 12-transmembrane domain protein present in the ER. In mice, UNC-93B delivers the nucleotide-sensing receptors TLR3, 7, and 9 from the ER to endolysosomes (Fig. 36.1; Kim et al., 2008). Three individuals, from two European countries (Portugal and France) and originating from two consanguineous kindreds of Gypsy origin, have been found to carry homozygous mutations in *UNC93B1*. A homozygous 1034del4 mutation is carried by one HSE patient of Portuguese origin, and a homozygous 781G>A mutation was identified in two individuals from a Gypsy family living in France, one of whom developed HSE (Fig. 36.10; Casrouge et al., 2006, and unpublished data). Both mutations created a frameshift and a premature stop codon in the mRNA of *UNC93B1*. The leukocyte and fibroblast cells of patients with UNC-93B deficiency do not respond to TLR3, 7, 8, or 9 agonists in terms of IFN-α, IFN-β, and IFN-λ production, in particular. Impaired TLR7, 8, and 9 responses probably play no more than a moderate role in the pathogenesis of HSE, as patients with IRAK-4 and MyD88 deficiencies do not respond to TLR7, TLR8, or TLR9 either but did not suffer from HSE (Picard et al., 2003; von Bernuth et al., 2008). The fibroblast cells of UNC-93B–deficient patients displayed abnormal high levels of viral replication and cell death after infection with HSV-1 or VSV. All immunological parameters, such as the numbers of blood phagocytes, B and T lymphocytes, and NK cells, lymphocyte subset distribution (CD4 T, CD8 T), antibody production, and T-cell proliferation in response to mitogens and antigens tested, were normal in the two patients evaluated.

Clinical and Pathological Manifestations and Treatment

Two of the three UNC-93B–deficient individuals developed HSE. The first patient presented with his first episode of HSE at the age of 11 months, during HSV-1 primary infection. HSE was diagnosed on the basis of meningitis, necrotic bilateral temporal lesions on CT scan, and an increase in anti-HSV-1 antibody titers in the CSF. The patient was treated with IV acyclovir and initially recovered well. Three months later, encephalitis recurred, without the detection of HSV-1. IV acyclovir treatment was administered, followed by oral acyclovir for 2 months. At the age of 3.5 years, the patient presented a new episode of encephalitis, with coma and meningitis, together with new lesions on CT scan, but PCR tests for HSV-1 carried out on CSF were negative. IV acyclovir treatment was given for 1 month, leading to an improvement in his clinical status. During 14 years of follow-up, the patient has experienced no subsequent acute events. The second patient developed her first episode of meningoencephalitis at the age of 5 years. HSE was diagnosed on the basis of meningitis, right frontoparietal necrosis on MRI, and positive PCR tests for the presence of HSV-1 in CSF samples. The patient's clinical status improved on IV acyclovir treatment. She was admitted again at the age of 17 years for a second episode with meningitis, a strongly positive HSV-1 PCR, and intrathecal IFN-α production. Her clinical status improved with IV acyclovir therapy. She is now 21 years old and her clinical status has not worsened since the second episode of HSE. One sibling of the second patient, who carries the same homozygous 781G>A mutation in *UNC93B1*, is now 30 years old and did not develop HSE despite serologically documented HSV-1 infection. The two patients with UNC-93B deficiency and HSE have been exposed to other viruses without obvious clinical manifestations, including CMV, VZV, EBV, HHV6 parvovirus B19, respiratory syncytial virus, and parainfluenza-1, influenza A, and B viruses. They have also been immunized with live mumps/rubella/measles and poliovirus vaccines with no adverse effect (Casrouge et al., 2006).

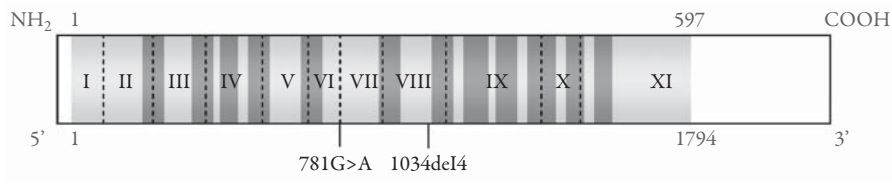

Figure 36.10 *Schematic diagram of the structure of the UNC93B1 gene and the mutations identified.* Human *UNC93B1* has 11 exons (Roman numerals), encoding a protein with 12 predicted transmembrane domains (shown in gray). The position of all mutations reported is shown in nucleotide nomenclature at the bottom.

HUMAN TRAF3 DEFICIENCY

Molecular Basis and Laboratory Findings

A French patient with autosomal dominant tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) deficiency and HSE has recently been identified (Perez de Diego et al., 2010). TRAF-3 has functions downstream of multiple TNF receptors as well as downstream of TLR-3 and controls IFN-α, IFN-β, and IFN-λ production (Fig. 36.1). The de novo germline heterozygous R118W missense mutation results in loss of expression, loss of function, and a dominant-negative effect (Fig. 36.11). Leukocytes and fibroblasts from the patient displayed impaired responses to TLR3 agonist stimulation, in terms of IFN-β and IFN-λ production, in particular. The fibroblast cells of the patient displayed higher levels of viral replication and cell death after VSV infection than the control cells. Monocyte-derived dendritic cells and monocyte-derived macrophages from the patient were found to produce only small amounts of IFN-α, IL-12p40, TNF-α, and IL-6 in response to stimulation with TLR4 and TLR7–8 agonists. The responses to activation with RIG-I and MDA-5 in the patient's SV40-fibroblast cells also seemed to be partially impaired. Moreover, TRAF3 deficiency affects the TNF-R pathways, including the CD40, LTβR, and BAFF-R pathways. However, the lack of a corresponding overt clinical phenotype in the patient is consistent with an incomplete functional defect. All standard immunological parameters, such as the number of blood phagocytes, B and T lymphocytes and NK cells, lymphocyte subset distribution (CD4 T, CD8 T), antibody production, and T-cell proliferation in response to mitogens and antigens tested, were normal in the patient. In conclusion, various TRAF3-dependent pathways are impaired in the patient's cells, including the IFN-α/-β, -λ-inducing, and TNF-R-responsive pathways. However, residual TRAF3-dependent signaling is sufficient for most defects to remain clinically silent. By contrast, the impaired TLR3 response is symptomatic and causes HSE, implying that the TLR3 pathway is critically dependent on TRAF3 and essential for immunity to HSV-1 in the CNS.

Clinical and Pathological Manifestations and Treatment

The first clinical manifestations of HSE in the TRAF3-deficient patient appeared at the age of 4 years. The patient had persistent fever and convulsions, and HSV-1 was detected on CSF analysis. CT scan showed hypodensity in the left frontal temporal lobe. She was treated with IV acyclovir (60 mg/kg/d for 3 weeks) and initially recovered. The patient had chickenpox 1 to 2 months before HSE, her mother had cold sores, and one brother had also suffered from cold sores, viral meningitis, and gingivostomatitis, but none of her grandparents, parents, or brothers had developed HSE and none of these relatives carried the TRAF3 mutation. The patient with TRAF3 deficiency and HSE described here is now 18 years old and has otherwise remained healthy with no prophylaxis. She shows normal resistance to other infectious diseases, including viral diseases in particular, despite serologically documented EBV, VZV and HSV-2 infection.

HUMAN TRIF DEFICIENCY

Molecular Basis and Laboratory Findings

Two kindreds with childhood HSE associated with TRIF deficiency (Fig. 36.1) were recently identified (Sancho-Shimizu et al., 2011). The only affected member of family 1 is one of five offspring of consanguineous parents from Saudi Arabia and was found to have a homozygous nonsense mutation (R141X) in the N-terminal region of the *TRIF* gene resulting in complete absence of protein. Most of the other family members were heterozygous for the R141X mutation and none had a history of encephalitis. This loss of expression/function of TRIF resulted in the abolition of TLR3-mediated signaling and TRIF-dependent TLR4 responses as measured by IFN-β and IFN-λ production.

Patient 2, from a nonconsanguineous European family, has a heterozygous missense mutation (S186L) in the N-terminal part of the *TRIF* gene, suggesting that this hypomorphic S186L allele of TRIF is dominant negative and responsible for autosomal dominant TRIF deficiency resulting in HSE, although with incomplete penetrance as only one of the three S186L TRIF heterozygotes (mother and maternal grandfather) developed HSE following HSV-1 infection (Sancho-Shimizu et al., 2011).

Clinical and Pathological Manifestations

The affected boy from family 1 with the homozygous R141X mutation had no infectious problems until age 2, when he developed HSE complicated by seizures, EEG abnormalities, atrophy of the left temporal lobe, and delayed speech. The

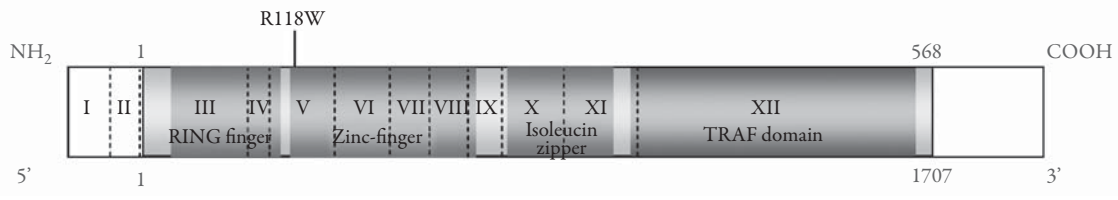

Figure 36.11 *Schematic diagram of the structure of the TRAF3 gene and the mutations identified.* Human *TRAF3* has 12 exons (Roman numerals), encoding a protein with a ring-finger and five zinc-finger domains in the N-terminal region, followed by an isoleucine zipper and a TRAF domain in the C-terminal region. The position of the mutations reported is represented with amino-acid nomenclature at the top.

affected offspring of family 2 was healthy until she developed HSE at age 21 months. She responded to treatment with acyclovir and never relapsed but has neurologic sequelae (blindness and epilepsy).

CONCLUSIONS

Human IRAK-4 and MyD88 deficiencies cause a selective predisposition to pyogenic bacterial diseases (mainly caused by *S. pneumoniae*, *S. aureus*, and *P. aeruginosa*) in early childhood but become asymptomatic after adolescence. Autosomal recessive mutations in the *IRAK4* or *MYD88* gene selectively impair TLRs other than TLR3 and most IL-1R responses. The MyD88- and IRAK-4–dependent pathway is essential for protective immunity to a few pyogenic bacteria in childhood but is otherwise redundant in host defense. X-linked hypomorphic mutations in *NEMO* and autosomal hypermorphic mutations in *IKBA* impair NF-κB–mediated signaling, at least in response to the stimulation of TLRs, IL-1Rs, and TNF receptors, and confer a broad predisposition to infections and PIDs with no improvement during life. Finally, clinical genetic studies have revealed that rare mutations in the TLR3-TRIF alternative pathway underlie HSE in otherwise healthy children: autosomal recessive UNC-93B, autosomal dominant and recessive TLR3, autosomal dominant TRAF3, and autosomal recessive and dominant TRIF deficiencies. These six disorders confer predisposition to HSE, consistent with the abundant and almost selective expression of TLR3 in resident cells from the CNS, including neurons, oligodendrocytes, astrocytes, and microglial cells (Lafaille et al. 2012). The lack of TLR3 responses results in the impaired production of antiviral IFN-α/-β and -λ, enhanced viral replication, and high levels of cell death. It seems likely that new inborn errors of immunity in these NF-κB–involved pathways will soon be discovered in patients with these or other infectious diseases (Alcais et al., 2010; Casanova and Abel, 2007; Casanova et al., 2011).

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37.

CARTILAGE-HAIR HYPOPLASIA

Outi Mäkitie

DESCRIPTION

Cartilage-hair hypoplasia (CHH), or metaphyseal chondrodysplasia, McKusick type (MIM 250250), is an autosomal recessive skeletal dysplasia in which immunodeficiency is a constant feature (McKusick et al., 1965). Skeletal dysplasias comprise a large group of clinically distinct and genetically heterogeneous conditions characterized by abnormalities in patterning, linear growth, differentiation, and maintenance of the human skeleton, beginning during the early stages of fetal development and evolving throughout life. In the 2006 revision of the International Nosology and Classification of Genetic Skeletal Disorders, 372 different conditions were listed in 37 groups defined by molecular, biochemical, and/or radiographic criteria (Superti-Furga and Unger, 2007). The metaphyseal chondrodysplasias constitute a subgroup of bone dysplasias with eight distinct disorders.

CHH is a pleiotropic skeletal dysplasia with symptoms arising also from several nonskeletal tissues (Makitie et al., 1995; Makitie and Kaitila, 1993; McKusick et al., 1965). In the original description of CHH, McKusick et al. observed an increased propensity to infections, caused by viruses in particular. Since then the defective immunity in CHH has been confirmed by clinical and laboratory studies (Makitie et al., 1998, 2000b; Polmar and Pierce, 1986). The disease-causing gene, *RMRP*, RNA component of mitochondrial RNA processing endoribonuclease, was identified in 2001, and a number of mutations have been found (Ridanpaa et al., 2001, 2002). However, the pathogenic mechanisms of the pleiotropic features, including the immunodeficiency, have remained elusive.

CHH is prevalent among the Amish in the United States and among the Finns in Europe, but affected families have been observed in most Caucasian and Asian populations (Nakashima et al., 2003; Ridanpaa et al., 2002).

CLINICAL MANIFESTATIONS

NONIMMUNOLOGICAL FEATURES

The clinical features include growth failure and hair hypoplasia (Makitie and Kaitila, 1993; Makitie et al., 1995), anemia (Juvonen et al., 1995; Makitie et al., 1992b), intestinal neuronal dysplasia or Hirschsprung's disease (HD) (Makitie et al., 2001a), defective spermatogenesis (Makitie et al., 2001c), and risk of malignancies (Makitie et al., 1999) (Table 37.1 and Fig. 37.1).

The marked short-limbed, short stature is due to metaphyseal dysplasia. The growth failure has its onset prenatally and progresses with age. In Finnish patients the mean birth length was 45.8 cm for boys and 44.9 cm for girls (range, 38–51 cm), and the median adult height was 131.2 cm (range, 110–158 cm) for males and 122.5 cm (range, 103–137 cm) for females (Makitie et al. 1992a; Makitie and Kaitila, 1993).

The radiographic skeletal abnormalities include short and broad tubular bones with splaying and an irregular metaphyseal border of the growth plate (Fig. 37.2). The costochondral junctions are similarly splayed and irregular; the vertebrae are usually normal. These findings develop and are diagnostic by the age of 6 to 9 months. In adults the tubular bones remain short and thick but are otherwise unspecific (Makitie and Kaitila, 1993; Makitie et al., 1995).

The characteristic hair hypoplasia in CHH presents as fair, thin, and sparse hair growth. However, variation is marked and individuals with normal hair have been observed (Bonafe

Table 37.1 PLEIOTROPIC FEATURES IN CARTILAGE-HAIR HYPOPLASIA

FEATURE	FREQUENCY (%)
Short stature, –4 SD or <5th percentile	100
Hair hypoplasia	93
Immunodeficiency	56
Propensity to infections	56
In vitro immunodeficiency	88
Hypoplastic childhood anemia	79
Gastrointestinal dysfunction	18
Hirschsprung's disease	9
Defective spermatogenesis	100
Metaphyseal chondrodysplasia on childhood skeletal radiographs	100
Overall risk of malignancies (standardized incidence ratio)	7.0
Non-Hodgkin's lymphoma (standardized incidence ratio)	90
Basal cell carcinoma (standardized incidence ratio)	33

Data from Makitie and Kaitila, 1993; Makitie et al. 1999, 2001b; Taskinen et al., 2008.

et al., 2002; Makitie and Kaitila, 1993; Makitie et al., 1995; Verloes et al., 1990).

Gastrointestinal problems, such as neuronal dysplasia of the intestine, are common in CHH. Congenital HD was found in 13 of 147 Finnish patients with CHH (9 percent), all of whom had an overall severe form (Makitie et al., 2001a). Eight patients had the classic form of HD with rectosigmoid involvement, two had long-segment colonic disease, and three had total colonic aganglionosis. Six of the patients had episodes of enterocolitis, two with colonic perforations prior to the first surgery; 11 patients had postoperative enterocolitis and some died of enterocolitis-related septicemia (Makitie et al., 2002).

Defective erythrogenesis in early childhood presenting as refractory hypoplastic anemia is a common feature in CHH and was found in 54 of 74 Finnish patients (73 percent). In approximately 6 percent of CHH patients severe anemia is persistent and resembles Diamond-Blackfan anemia (Makitie et al., 1992b, 2000a; Williams et al., 2005). Thrombocytopenia and autoimmune hemolytic anemia have also been reported (Ashby and Evans, 1986; Berthet et al., 1996).

IMMUNOLOGICAL FEATURES

McKusick et al. (1965) observed an increased rate of infections in patients with CHH. Varicella occasionally resulted in a prolonged and severe disease with hemorrhagic vesicles, high fever, and even fatality. They showed in two Amish

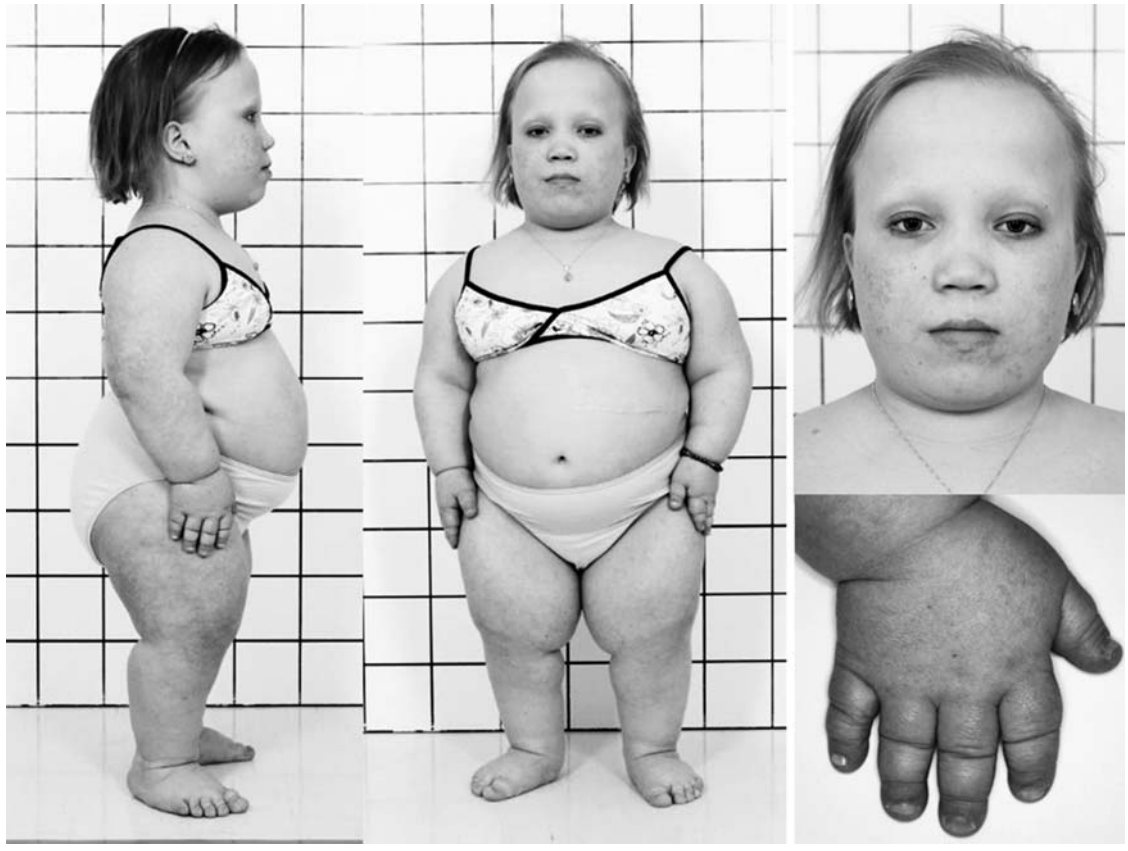

Figure 37.1 Patient with CHH. (See Color Plate.)

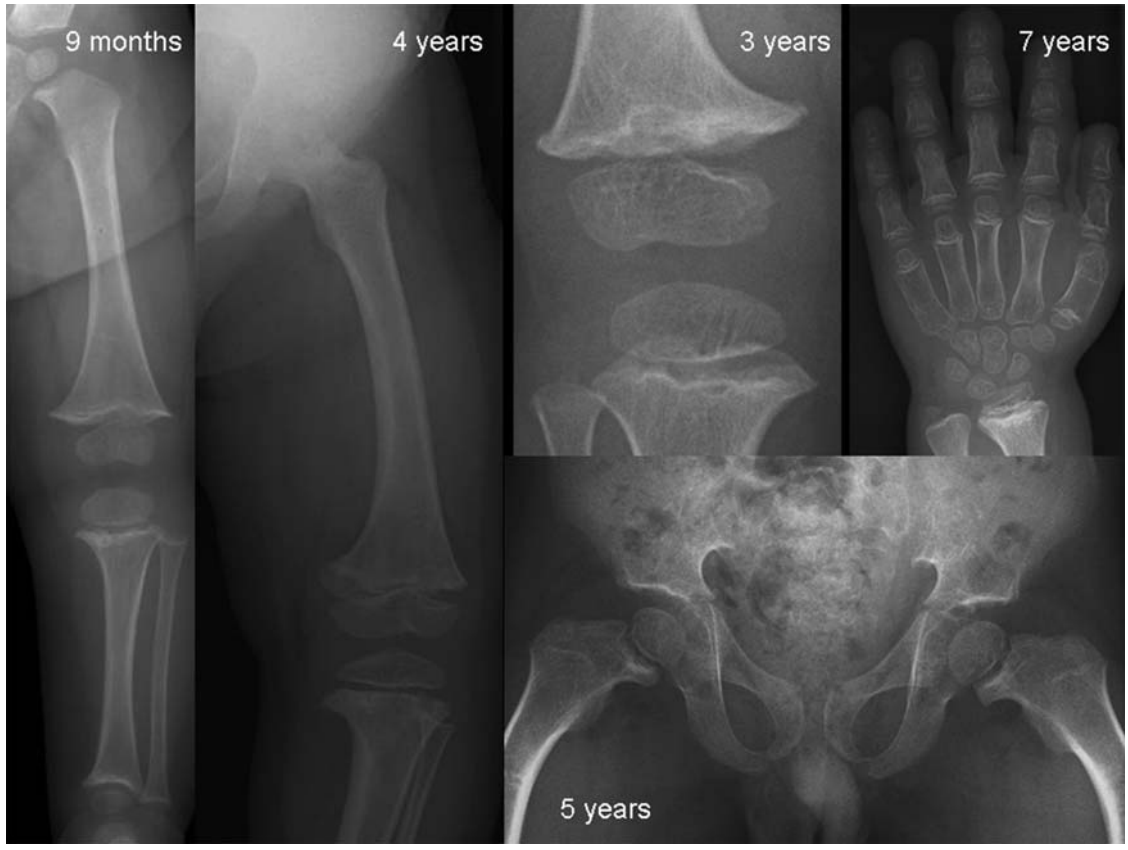

Figure 37.2 Radiographs showing the characteristic features. (See Color Plate.)

patients mild to moderate lymphopenia, decreased delayed hypersensitivity, and impaired lymphocyte responses to mitogens, whereas immunoglobulin concentrations and antibody synthesis were normal (Lux et al., 1970). Subsequent studies confirmed cell-mediated immune deficiency to be an integral feature of CHH (Pierce and Polmar, 1982; Pierce et al., 1983; Polmar and Pierce, 1986; Ranki et al., 1978; Rider et al., 2009; Trojak et al., 1981; Virolainen et al., 1978). The absolute lymphocyte count was about half that of normal controls. The results of allogeneic stimulations indicated an intrinsic T-cell defect, whereas antigen-presenting cells were not affected. The IL-2 production by CHH lymphocytes was reduced, but exogenous IL-2 did not correct the defect in proliferation (Kooijman et al., 1997; Pierce and Polmar, 1982; Pierce et al., 1983; Polmar and Pierce, 1986).

In the Finnish patients, a reduction of 50 percent in the $CD4^+$ cell count and a reduction of 30 percent in the $CD4^+/CD8^+$ cell ratio have been reported. The B-lymphocyte count was usually normal, whereas the NK-cell count was often elevated (Makitie et al., 1998). On the basis of increased expression of Fas (CD95), CD95L, and Bax, and decreased expression of Bcl-2 and inhibitor of apoptosis protein (IAP) in both $CD4^+$ and $CD8^+$ cells, it has been suggested that the lymphopenia might be due to increased apoptosis of these cells (Yel et al., 1999). In another study, the levels of mRNA encoding c-myc, IL-2Rα, IL-2, and IFN-γ were decreased in stimulated CHH lymphocytes, whereas those of other early activation gene products, such as c-fos and c-jun, were not impaired, suggesting a lymphocyte intracellular signaling defect (Castigli et al., 1995). Transcriptional profiling of CHH patient RNAs identified several upregulated and downregulated genes that play a role in the immune system, cell-cycle regulation, and signal transduction (Hermanns et al., 2005).

In contrast to earlier studies, we found that one third of the Finnish CHH patients also had partially defective humoral immunity presenting as isolated IgA and IgG subclass deficiencies (Makitie et al., 2000b; Toiviainen-Salo et al., 2008). Impaired antibody production has been observed (Rider et al., 2009). Several CHH patients with combined immune deficiency have been reported also from other populations (Guggenheim et al., 2006; Kavadas et al., 2008; Rider et al., 2009).

A number of patients with chronic, severe, and even fatal infections due to viruses, bacteria, and fungi have been reported (Berthet et al., 1996; Castigli et al., 1995; Guggenheim et al., 2006; Hong, 1989; Kainulainen et al., 2008; Lux et al., 1970; Polmar and Pierce, 1986; Saulsbury et al., 1975; Steele et al., 1976). CHH patients, particularly those with defective humoral immunity, have an increased risk for bronchiectasis (Toiviainen-Salo et al., 2008). Despite severe clinical presentations in occasional patients, most patients do clinically relatively well (Makitie et al., 1998, 2000b; Rider et al. 2009).

MOLECULAR BASIS

On the basis of molecular linkage studies on Finnish multiplex CHH families, the disease-causing gene was mapped to

9p12 in 1993 (Sulisalo et al., 1993). The location was refined by disequilibrium analysis; finally, the mutated gene, *RMRP*, was detected through physical mapping and sequencing (Ridanpaa et al., 2001). Several *RMRP* mutations have been detected (Bonafé et al., 2005; Ridanpaa et al., 2002). Most are base substitutions, insertions, or short duplications that alter conserved nucleotide sequences in the transcribed region. Insertions or duplications in the promoter region between the TATA box and the site of initiation of transcription, or in the 5′ end of the transcribed region, are also common.

The most common CHH-causing mutation is 70A > G, found in 92 percent of Finnish CHH patients and probably all Amish patients (Ridanpaa et al., 2003). The same mutation accounts for 48 percent of the mutations among patients from other parts of Europe, North and South America, the Near East, and Australia (Ridanpää et al., 2001, 2002).

The human *RMRP* gene encodes the 267 bp RNA molecule of the RNase MRP complex, which consists of protein components and the RNA molecule. Thus, unlike most of the known disease-associated genes, the *RMRP* is an untranslated gene. The RNase MRP is a ribonucleoprotein endoribonuclease involved in the processing of precursor ribosomal RNA and in priming of the RNA for mitochondrial DNA replication. It has been suggested that the RNase MRP also carries other important biological functions, such as control of cell proliferation (Clayton, 2001; Maida et al., 2009). In situ hybridization experiments have indicated the presence of RNase MRP in both mitochondria and nucleoli, the majority being localized to the latter. The pathogenetic mechanisms of the *RMRP* mutations are still unknown.

TREATMENT AND PROGNOSIS

Children with CHH should not be vaccinated with live or attenuated bacteria or viruses. Antibiotic and antiviral treatment of infections, as well as prophylactic antibiotics, should be recommended on a case-by-case basis. Immunoglobulin treatment is indicated in patients with combined immune deficiency.

Profound T-cell deficiency similar to that seen in severe combined immunodeficiency has been demonstrated in some patients with CHH. Anecdotal CHH patients with such severe presentation have undergone bone marrow transplantation with successful long-term reconstitution of immunity; no improvement was observed in longitudinal growth, however (Berthet et al., 1996; Guggenheim et al., 2006)

Patients with CHH have significantly increased mortality rates compared with their parents and nonaffected siblings (Makitie et al., 2001b). While infections predispose younger children to premature death, malignancies predominate as the cause of death in the older age groups (Makitie et al., 1999, 2001b). A recent follow-up study including 123 Finnish CHH patients identified 14 cases of cancer (standardized incidence ratio [SIR] 7.0). Non-Hodgkin's lymphoma was the most frequent cancer type (9 patients, SIR 90.2). Nine of the 14 cancers were diagnosed in patients less than 45 years of age. In addition, 10 patients had basal cell carcinoma of the skin (SIR 33.2) (Taskinen et al., 2008). Kaplan-Meier estimation of cancer events gave a probability of a cancer event (excluding basal cell carcinoma) of 41 percent by the age of 65 years (Taskinen et al., 2008).

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38.

HYPER-IGE RECURRENT INFECTION SYNDROMES

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Hyper-immunoglobulin E recurrent infection syndrome (HIES; MIM #147060, #243700) has been recognized as a primary immunodeficiency characterized by recurrent staphylococcal skin abscesses, pneumonias with pneumatocele formation, extreme elevations of serum IgE, eosinophilia, and distinct abnormalities of the connective tissue, skeleton, and dentition (Belohradsky et al., 1987; Buckley et al., 1972; Davis et al., 1966; Donabedian and Gallin, 1983a; Erlewyn-Lajeunesse, 2000; Grimbacher et al., 1999a; Hill and Quie, 1974). The majority of cases of autosomal dominant HIES (AD-HIES; MIM #147060) are caused by mutations in signal transducer and activator of transcription 3 (STAT3) (Holland et al., 2007; Jiao et al., 2008; Minegishi et al., 2007; Renner et al., 2008). A distinct hyper-IgE syndrome, described as autosomal recessive hyper-IgE syndrome (AR-HIES; MIM #243700), was described by Renner et al. in 2004. Mutations in DOCK8 account for the majority of these patients, characterized by sinopulmonary infections, viral infections of the skin, allergy, and predisposition to malignancy (Engelhardt et al., 2009; Zhang et al., 2009).

AD-HIES

HISTORY

"So went Satan forth from the presence of the Lord, and smote Job with sore boils from the sole of his foot unto his crown." With this citation from Job II: 7, Davis, Schaller, and Wedgewood coined the term *Job syndrome* in 1966 (Davis et al., 1966). They reported two red-haired, fair-skinned girls who had frequent sinopulmonary infections, severe dermatitis, and recurrent staphylococcal skin infections that were remarkable for their lack of surrounding warmth, erythema, or tenderness. The syndrome was further defined and clarified by Buckley et al. (1972), who noted similar infectious problems in two boys with severe dermatitis, distinctive facial appearance, and elevated IgE levels, leading to the term *Buckley syndrome*. Following this report, elevated levels of IgE and a defect in neutrophil chemotaxis were reported in the two girls from the initial report (Hill and Quie, 1974), showing that Job syndrome and Buckley syndrome represented the same condition. To avoid further confusion, the name *hyper-immunoglobulin E recurrent infection syndrome* (HIES) is now widely used. In 1999 a group at the U.S. National Institutes of Health (NIH) undertook to define further the phenotype of HIES, noting that the syndrome was heritable as an autosomal dominant condition (hence referred to in this chapter as AD-HIES, even though many cases are sporadic due to new mutations, as discussed below) with both immune and nonimmune features.

CLINICAL PRESENTATION

The clinical features of AD-HIES include the immune system, the connective tissues and skeleton, the vasculature, and dentition. Table 38.1 shows the frequency of the most consistent clinical and laboratory findings in AD-HIES. These 19 clinical features associated with AD-HIES were published based on a cohort of 30 patients with the disease (Grimbacher et al., 1999a) and have since been confirmed in many more individuals. Multivariate analysis of these features in AD-HIES patients revealed that IgE greater than 1,000 IU plus five of the features—recurrent pneumonia, newborn rash, pathological bone fractures, characteristic face, and high palate—were highly indicative of a STAT3 mutation (Woellner et al., 2010).

Table 38.1 INCIDENCE OF CLINICAL FINDINGS ASSOCIATED WITH HYPER-IGE RECURRENT INFECTION SYNDROME

	% of cases
Findings Related to Infections and Immune System	
Moderate to severe eczema	100
Serum IgE >2,000 IU/mL	97
Eosinophilia (>2 SD above normal mean)	93
Recurrent pneumonia, X-ray proven	85
Recurrent skin abscesses	85
Recurrent (>3 per year) upper respiratory infections	80
Mucocutaneous candidiasis	80
Pneumatoceles	75
Newborn rash	75
Other serious infections	50
Lymphoma, other cancer	3
Findings Related to Bones, Teeth, and Connective Tissue	
Characteristic face	80
Retained primary teeth	70
Hyperextensibility of joints	70
Increased nasal width (interalar distance >1 SD above normal value for age, race)	65
Scoliosis >10 degrees	60
Recurrent fractures following minor trauma	55
High-arched palate	50
Congenital skeletal anomalies	10
Focal hyperintensities on brain MRI	80
Chiari type I malformation	40
Coronary vasculature abnormalities	60

IMMUNE SYSTEM

Eczema, abscesses, pneumonia, mucocutaneous candidiasis, elevated serum IgE, and eosinophilia are the most common features of immunodeficiency and immune dysregulation in AD-HIES patients. The initial presentation is a newborn rash, which may even be present at birth (Color Plate 38.I). Scrapings of this rash typically show eosinophils. The newborn rash may improve or progress to an eczematoid dermatitis. The dermatitis of AD-HIES is usually driven by *Staphylococcus aureus* and improves with control of *S. aureus* with antistaphylococcal antibiotics or antiseptics. The dermatitis is in a distribution somewhat atypical for atopic dermatitis, involving the posterior auricular areas, back, buttocks, and scalp (Color Plate 38.II). Furuncles or boils are almost universal in AD-HIES unless staphylococcal colonization is well controlled with prophylactic antibiotics and/or antiseptics. The boils may be "cold" and nontender and are usually not associated with fever or signs of local or generalized inflammation (Erlewyn-Lajeunesse, 2000). However, despite the outward appearance of diminished inflammation, these abscesses are typically filled with pus and almost always grow *S. aureus*. Surgical drainage of these lesions is commonly required, and they typically resolve without spreading through the dermis and fascia. With diagnosis earlier in life and institution of prophylactic antibiotics, we have seen mostly impetiginized eczema and fewer boils than previously described. In our cohort, 85 percent of AD-HIES patients had recurrent skin abscesses, and over half had a history of more than four abscesses.

Sinopulmonary infections are frequent in AD-HIES. Individuals have a high palate, which in addition to the immunodeficiency may interfere with clearance of sinus and ear drainage and lead to chronic infections. The majority of patients report four or more episodes of upper respiratory tract infections such as sinusitis per year.

A clinical hallmark of AD-HIES is recurrent pneumonia. The great majority of patients have had at least one bout of pneumonia in their lifetime, but more than half have had three or more. The most common pathogens of acute pneumonia are *S. aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*. For reasons still unclear, lung parenchymal abnormalities frequently complicate these pneumonias. About three quarters of AD-HIES patients develop long-term pulmonary complications, including bronchiectasis and pneumatoceles (Fig. 38.1). The cycle of infection and lung destruction is further exacerbated by superinfection of the cavities and bronchiectatic lung with *Pseudomonas aeruginosa* and *Aspergillus fumigatus*. The pulmonary fungal and *Pseudomonas* superinfections are a major source of morbidity and mortality in this disease (Freeman et al., 2007). Pulmonary infections less commonly seen in HIES include *Pneumocystis jiroveci* (Freeman et al., 2006), which may occur in infancy as the first lung infection, and nontuberculous mycobacteria (Melia et al., 2009).

Chronic candidiasis of mucosal sites and the nail beds affects about 80 percent of AD-HIES patients, including children (Color Plate 38.III). Although primary invasive fungal infections are relatively uncommon, focal extrapulmonary infections with *Cryptococcus* and *Histoplasmosis*, such as of the genitourinary tract and gut, occur infrequently (Hutto et al., 1988; Jacobs et al., 1984).

SKELETAL AND CONNECTIVE TISSUE ANOMALIES

Skeletal and facial abnormalities associated with HIES were recognized in the original reports by Davis et al. (1966) and Buckley et al. (1972). They noted characteristic facies and hyperextensibility of the joints. The facies in HIES are often asymmetrical, with a prominent forehead and mild prognathism, increased interalar width of the nose, wide-set eyes, thickening of the soft tissue of ears and nose, and a high-arched palate (Borges et al., 1998; Grimbacher et al., 1999a). The facial features progressively develop over childhood and become almost universal by late adolescence (Color Plate 38.IV). Sixty percent have an increased interalar distance (width across the bottom of the nose) and almost half have a high-arched palate

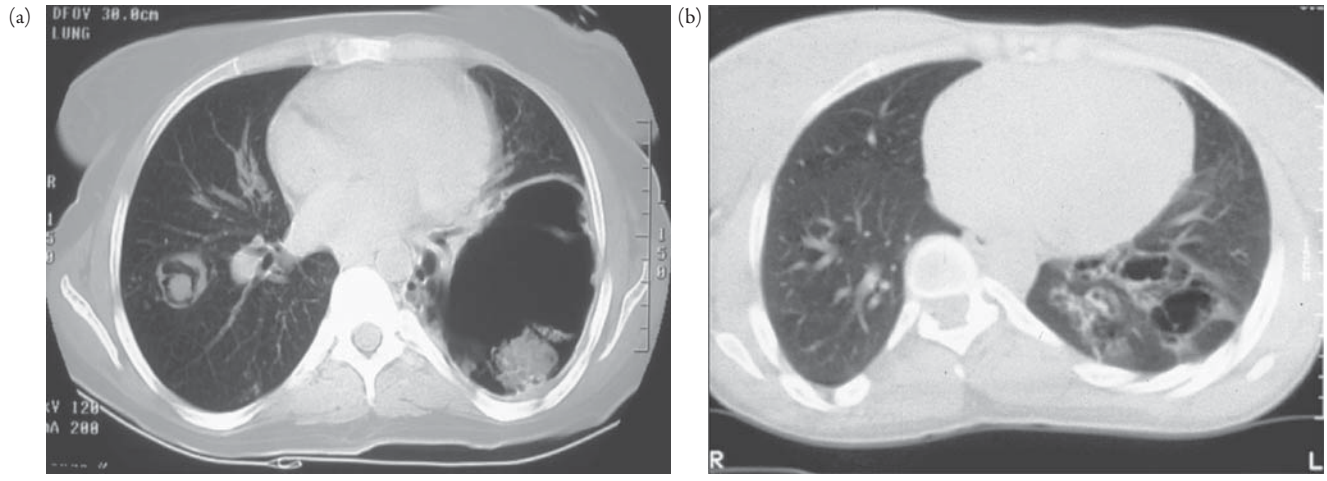

Figure 38.1 Pulmonary complications of hyper-IgE syndrome. (a) Postinflammatory cysts containing aspergillomas. (b) Scoliosis evident in angle of vertebra. (c) Degenerative spine disease in an adult with HIES.

(Color Plate 38.V, top right). Craniosynostosis has also been reported in a few cases of HIES (Gahr et al., 1987; Höger et al., 1985; Smithwick et al., 1978), and Chiari I malformations occur in about 20 percent of patients (Freeman et al., 2007). The Chiari malformations have been largely asymptomatic and have not required surgical correction.

Recurrent fractures with only minimal trauma occur in about half of patients, confirming the multisystem nature of AD-HIES. Fractures occur most commonly in the long bones and ribs (Fig. 38.2A). Although osteopenia is common and may be cytokine driven (Cohen-Solal et al., 1995; Leung et al., 1988), the fractures that occur are not at the same sites typical of osteoporosis in postmenopausal females and the osteopenia does not correlate directly with the number of minimal-trauma fractures. Scoliosis has been seen in about 60 percent of patients: over half had a curvature of more than 15 degrees, and one third had more than 20 degrees (Fig 38.2B). Although many cases of scoliosis are idiopathic, diverse contributing factors have been noted, such as leg-length discrepancy, prior thoracotomy (e.g., for lung cyst removal), and vertebral body anomalies. Several patients have had severe scoliosis requiring surgical correction with rod placement. Hyperextensibility of the joints was noted in the initial reports and was found in 68 percent of our patients. Degenerative bone disease, primarily of the spine, often appears with increasing age and can result in significant pain (Fig. 38.2C). Spinal fusions and other surgical procedures have ameliorated symptoms for some individuals.

DENTITION AND ORAL FINDINGS

In our cohort of AD-HIES patients older than age 6, about 70 percent had delayed shedding of three or more primary teeth (Color Plate 38.V). Reduced resorption of primary tooth roots may lead to prolonged retention of the primary teeth, which in turn prevents the appropriate eruption of the permanent successors (O'Connell et al., 2000). The mechanism underlying this unique abnormality is unknown. We currently recommend that, after checking by radiography for the existence and developmental age of the secondary teeth, children with AD-HIES undergo extraction of retained primary teeth. This procedure is usually followed by normal eruption of the permanent teeth. Abnormalities of oral mucosa have been described, including central ridges and fissures of the palate, central depressions of the tongue, and oral mucosal variants of the cheek (Domingo et al., 2008).

VASCULAR FINDINGS

Vascular abnormalities are increasingly recognized in AD-HIES. One individual was found to have a large coronary artery aneurysm after suffering a myocardial infarction (Ling et al., 2007). This finding and other reported cases of aneurysms led to more systematic imaging of coronary arteries (Alomar-Melero et al., 2008; Young et al., 2007). Abnormalities are common and manifest as tortuosity, dilation, and less frequently aneurysm of the coronary arteries. Aneurysms have also been reported in the brain, though these vessels have not been studied as closely as the coronary arteries. Brain magnetic resonance imaging has shown an increase in lacunar infarcts at a younger age than expected, as well as focal hyperintensities of unclear etiology frequently in all age groups (Freeman et al., 2007).

OTHER ASSOCIATED CONDITIONS

Several malignancies have been reported in AD-HIES, which suggests that these patients may be at increased risk of both lymphomas and other tumors (Bale et al., 1977; Buckley and Sampson, 1981; Einsele et al., 1990; Gorin et al., 1989; Huber et al., 1987; Kowalchuk, 1996; Nester et al., 1998; Takimoto et al., 1996). In the NIH cohort, two patients developed non-Hodgkin's lymphoma and both were successfully treated; another was successfully treated for nodular sclerosing Hodgkin's disease. One patient developed metastatic squamous cell carcinoma of the tongue. Autoimmune phenomena such as systemic lupus erythematosus and rheumatoid arthritis occur infrequently in HIES (Brugnoni et al., 1998; Leyh et al., 1986; North et al., 1997; Schopfer et al.,

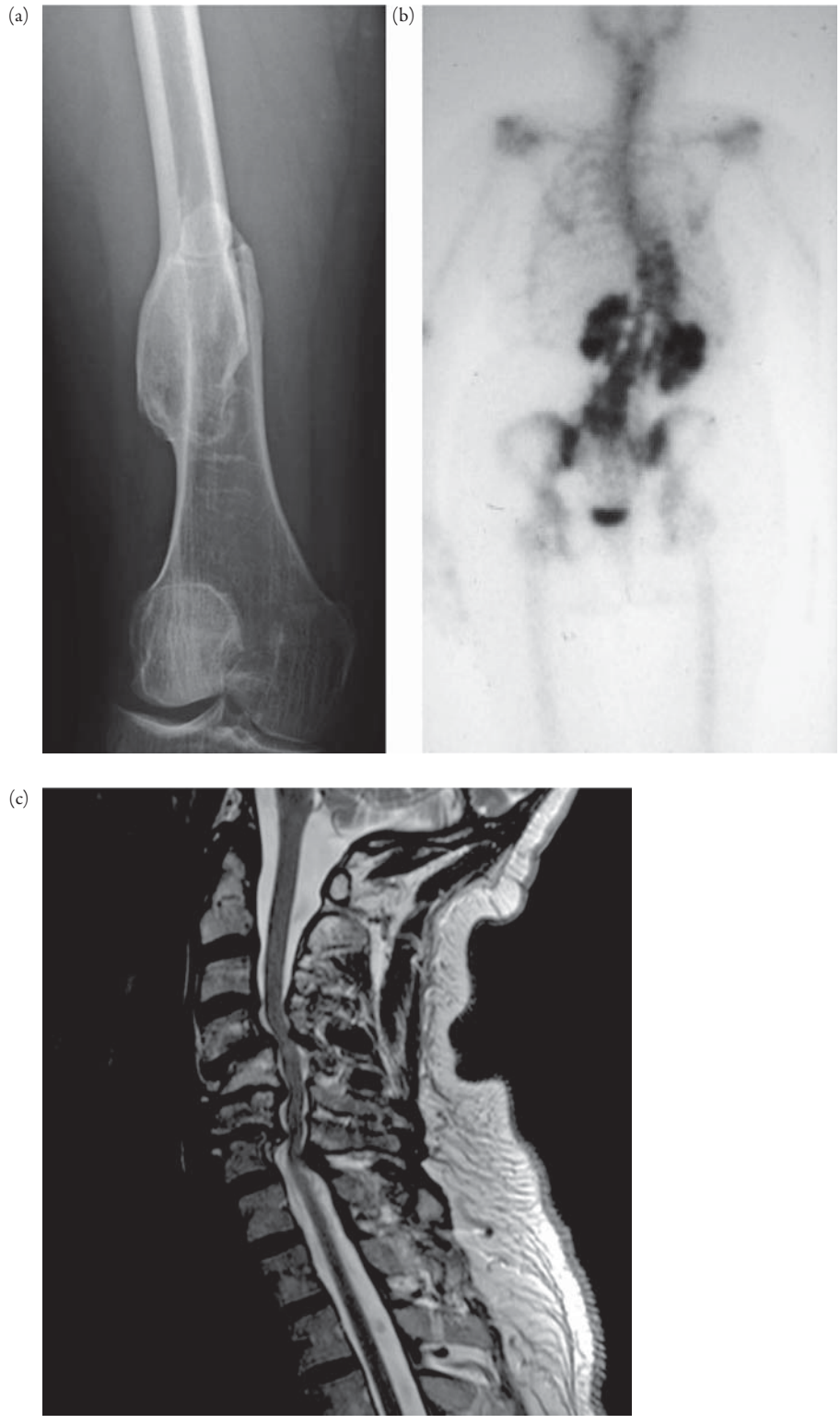

Figure 38.2 Skeletal manifestations of Job syndrome. (a) Healed pathological distal femoral fracture suffered while wading in the ocean. (b) Bone scan showing marked scoliosis. (c) Cervical spine magnetic resonance image of a 53-year-old with AD-HIES, demonstrating severe degenerative changes and spinal column narrowing.

1983). Autoimmune vasculitis (Kimata, 1995), dermatomyositis (Min et al., 1999), and membranoproliferative glomerulonephritis (Tanji et al., 1999) have been described in reports preceding genetic diagnosis, and may occur, but likely very infrequently.

LABORATORY FINDINGS

The syndrome takes its name from marked polyclonal elevations of IgE in serum, with an IgE of more than 2,000 IU/mL often being used as an arbitrary diagnostic level. However,

because IgE levels are very low in utero and climb only after birth in normal infants, infants affected with AD-HIES may not achieve a diagnostic level of 2,000 IU/mL early in life, and the levels may normalize or significantly decrease in adulthood (Grimbacher et al., 1999a). In affected infants the IgE is consistently elevated over the age-adjusted value, and a level 10 times the age-appropriate level has been a reasonable guide. Serum IgE levels are not static, and substantial fluctuations in serum IgE concentrations have been noted over time without any obvious relationship to the clinical status. Therefore, although a high IgE level is part of the diagnostic criteria for HIES, authentic cases may lack this particular feature at some point in their course, and the level of serum IgE apparently is not correlated with disease activity or severity. IgE production is thought to be elevated because of an increased number of B cells making subnormal levels of IgE (Garraud et al., 1999; King et al., 1991) and potentially impaired catabolism (Dreskin et al., 1987a). But the driving force for this abnormality is still unclear. In addition, the tropism of the elevated IgE is still unclear. Anti–*S. aureus* and anti–*Candida albicans* IgE titers have been noted to be raised in HIES, but any relationship to the severity of the disease is not known (Berger et al., 1980; Walsh et al., 1981). Unfortunately, raised anti-staphylococcal IgE titers are also seen in atopic patients and are therefore not helpful in the diagnosis of HIES. Anaphylaxis and allergies in general are not thought to be increased in AD-HIES.

White blood cell counts are typically in the normal range but have been reported to range from 1,700 to 60,000/μL (Buckley and Becker, 1978; Donabedian and Gallin, 1983a). Chronic leukopenia with borderline neutropenia has been observed in several patients (Donabedian and Gallin, 1983a). Eosinophilia is seen in more than 90 percent of HIES patients. This eosinophilia is at least 2 standard deviations above normal, usually above 700 cells/μL, may fluctuate over time, and is not correlated with the extent of IgE elevation.

DIAGNOSIS

An HIES scoring system (Table 38.2) was initially developed at NIH to assist in gene linkage studies (Grimbacher et al., 1999). Because for the majority of cases of AD-HIES the genetic etiology is now known to be mutations in STAT3, an alternative STAT3 score has been put forward (Woellner et al., 2010) to distinguish between STAT3 mutated and STAT3 wild-type patients. Individuals who have a similar phenotype but do not have a STAT3 mutation may still be classified with the original NIH scoring system, which is based on the frequency and severity of characteristics exhibited. In contrary to the STAT3 score, it includes an age correction, since older patients are likely to have collected more points, and some clinical features, such as shedding of primary teeth, cannot be evaluated in very young patients. For the NIH system, AD-HIES is considered highly likely if the score exceeds 40 points and unlikely if the score is below 20 points. Between 20 and 40 points is an indeterminate zone: patients with these scores may be suspected of having AD-HIES and followed over time to gather more conclusive data, or they may have other genetic forms of HIES (see below). If both immunological and nonimmunological features are present and contribute to the score, there is an increased likelihood of AD-HIES due to STAT3 mutations.

ETIOLOGY

The majority of cases of AD-HIES are caused by dominant-negative mutations in STAT3 (Holland et al., 2007; Jiao et al., 2008; Minegishi et al., 2007; Renner et al., 2008; Woellner et al., 2010). Most mutations are missense, resulting in one amino acid change, or short in-frame deletions. Mutations are largely concentrated in the SH2 and DNA binding domains of STAT3, with several mutational hot spots in each region. The SH2 domain mediates protein–protein binding, and the DNA binding domain mediates protein–DNA interactions. Despite the difference in functions of the two regions, and some differences in vitro in their effects on STAT3 signaling, clinically significant genotype–phenotype associations are absent.

Although HIES is thought to be rare, the exact incidence is unknown. Over 200 cases have been published. Although first described in Caucasians with red hair, STAT3 mutations have been found in all races and many ethnic groups, with equal frequency between genders. Transmission is autosomal dominant, with many sporadic cases reported. Penetration of the mutation is complete, and all family members carrying a specific mutation have the HIES phenotype, although there may be a variation in severity and phenotypic expressivity. Laboratory constructs of STAT3 mutations demonstrate a dominant-negative effect on STAT3 functioning. This is supported by the lack of null alleles in HIES, and the fact that mice with a complete heterozygous deletion of only one STAT3 allele are phenotypically normal. It is also noteworthy in this context that complete STAT3 knockout mice die in utero (Takeda et al., 1997).

IMMUNOLOGY AND PATHOGENESIS

The pathogenesis of both the immunological and nonimmunological features of AD-HIES are still not well understood. The most consistent immunological findings in HIES have been the elevated serum IgE, eosinophilia, diminished memory T- and B-cell populations, and greatly diminished Th17 cells and IL-17 production ex vivo (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Speckmann et al., 2008). Many studies have also focused on other cytokine production and responsiveness, as well as neutrophil chemotaxis, with conflicting results.

STAT3 is one of the STAT family of major signal transducers and is involved in many diverse pathways, including cancer, wound healing, angiogenesis, and immunity. Many cytokines, including IL-6, IL-10, IL-21, IL-22, and IL-23, signal through STAT3 (Fig. 38.3). That both proinflammatory cytokines (such as IL-6) and anti-inflammatory cytokines (such as IL-10) signal through STAT3 likely explains in part the dichotomy of AD-HIES being a disease of both too much and too little inflammation, as it is characterized by the exuberant local inflammation found in abscesses and pneumonia but the lack of systemic signs of illness such as fever and acute phase reactants (C-reactive protein, CRP).

Table 38.2 NIH HYPER-IGE SYNDROME SCORING SYSTEM

POINTS										
CLINICAL FINDING	0	1	2	3	4	5	6	7	8	10
Highest IgE (IU/mL)	<200	200–500			501–1,000				1,001–2,000	>2,000
Skin abscesses (total)	None		1 or 2		3 or 4				>4	
Pneumonias (X-ray proven, total)	None		1		2		3		>3	
Parenchymal lung abnormalities	Absent						Bronchiectasis		Pneumatocele	
Other serious infection	None				Severe					
Fatal infection	Absent				Present					
Highest eosinophils/μL	<700			700–800			>800			
Newborn rash	Absent				Present					
Eczema (worst stage)	Absent	Mild	Moderate		Severe					
Sinusitis, otitis (no. of times in worst year)	1 or 2	3	4–6		>6					
Candidiasis	None	Oral, vaginal	Fingernail		Systemic					
Retained primary teeth	None	1	2		3				>3	
Scoliosis, maximum curvature (degrees)	<10		10–14		15–20				>20	
Fractures with little trauma	None				1 or 2				>2	
Hyperextensible joints	Absent				Present					
Characteristic face	Absent		Mild			Present				
Increased nose width (interalar distance)	<1 SD	1–2 SD		>2 SD						
High palate	Absent		Present							
Congenital anomaly	Absent					Present				
Lymphoma	Absent				Present					

Leukocyte microarray data at rest and after stimulation and in vitro cytokine stimulation assays show increased levels of proinflammatory cytokines such as TNF-alpha and interferon-gamma (Holland et al., 2007; Yeganeh et al., 2008). Animal data support this finding, as mice with a conditional myeloid STAT3 knockout have increased proinflammatory cytokines (Takeda et al., 1999). However, in other aspects, AD-HIES is associated with diminished inflammatory cytokine and chemokine production. For instance, IL-6 leads to monocyte chemoattractant protein (MCP)-1 production, and this is disrupted in STAT3 deficiency, leading to decreased MCP-1 in vitro.

The immunodeficiency of STAT3 defects may be explained in part by a lack of differentiation of T lymphocytes into Th17 cells and subsequent IL-17 production. Both STAT3-deficient individuals as well as STAT3-deficient mice have impaired Th17-cell synthesis and IL-17 production. The role of IL-17 in human immunity is still being delineated, but mice with abnormal IL-17 signaling are prone to both *Candida* and extracellular bacterial infections (Aujla et al., 2008; Huang et al., 2004). In part, the immunity of IL-17 is thought to be due to regulation of IL-22, a key cytokine in human beta-defensin production (Dong et al., 2008). The antimicrobial peptide beta-defensin and neutrophil-recruiting chemokines, which are both important for protection against bacterial infection, are poorly produced by keratinocytes and lung epithelial cells when stimulated by T cells from HIES patients. Those cells, unlike other cell types, need the synergistic action of Th17 cytokines and classical proinflammatory cytokines to produce antibacterial factors. This might explain why HIES patients with impaired Th17 but normal proinflammatory cytokine production are susceptible to bacterial infections particularly in the skin and lung (Minegishi et al., 2009).

The greatly increased serum IgE in HIES remains poorly understood (Vercelli et al., 1990). Although IL-21 receptor knockout mice have elevated IgE, and IL-21 signals through STAT3, this finding does not appear to translate from mice to human, and impaired IL-21 signaling may actually result in less IgE in humans (Avery et al., 2008; Ozaki et al., 2002).

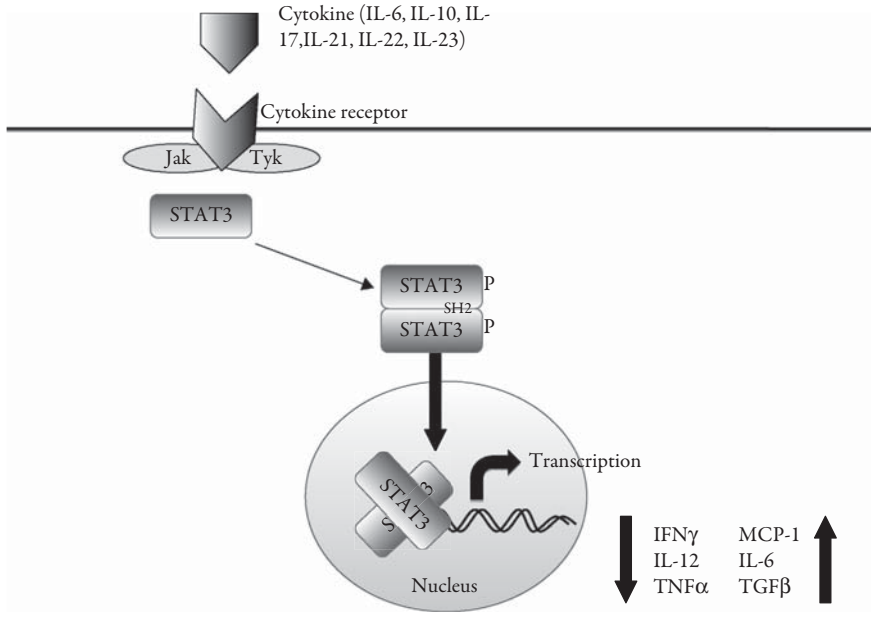

Figure 38.3 STAT3 signaling pathway. Multiple cytokines signal through transmembrane receptors that activate STAT3. The cytokine receptor activates the Janus kinase (Jak) family proteins leading to STAT3 phosphorylation and dimerization through the STAT3 SH2 domains. The STAT3 phosphodimer then enters the nucleus and binds to DNA to change the transcription program. Some of the regulatory effects of STAT3 are shown in the lower right corner.

B-cell development in mice with STAT3-deficient bone marrow have abnormal B-cell development, but again the translation into humans is not clear, and abnormal B-cell development has not been shown in AD-HIES. In general, people with AD-HIES have normal levels of B cells, normal-appearing lymph nodes, and normal IgG, IgM, and IgA. The only consistent B-lymphocyte abnormality has been deficient memory B cells and variable specific antibody responses (Speckmann et al., 2008).

PATHOGENESIS OF SOMATIC FINDINGS

How diminished STAT3 signaling leads to the many somatic features of HIES is still poorly understood. STAT3 knockout mice are embryologically lethal, but organ-specific STAT3 knockouts show evidence that STAT3 deficiency in mice does correlate with some of the somatic features. Mice with STAT3-deficient bone marrow have osteopenia with evidence of increased osteoclastogenesis, likely similar to the osteopenia and minimal-trauma fractures in AD-HIES (Zhang et al., 2005). Mice with STAT3-deficient astrocytes have evidence of astrocytosis and demyelination after brain injury, which may relate to the role of IL-6 in astrocyte development and be similar to the focal hyperintensities seen on brain magnetic resonance imaging in HIES (Datta et al., 2008; Okada et al., 2006). Mice with STAT3-deficient pulmonary epithelium have alveolar enlargement and excessive inflammation after injury, which may be similar to the residual pneumatoceles present in AD-HIES patients after lung infection (Hokuto et al., 2004). STAT3-deficient mouse keratinocytes have poor wound healing, which may be similar to the porous, doughy skin of human STAT3 deficiency as well as the oral mucosal variants (Sano et al., 1999). Other phenotypes of organ-specific STAT3 knockouts are not observed in humans, such as colitis and cardiomyopathy. The phenotype of transgenic mouse models with STAT3 mutations found in human AD-HIES is just being delineated and will add to our understanding of the pathogenesis of the somatic and immunological features.

TREATMENT

Aggressive skin care and prompt treatment of infections are the main pillars of management of all types of HIES. The dermatitis is heavily dependent on ongoing infection, typically with *S. aureus*. Therefore, antibiotic treatment is often an essential part of management of HIES eczema, in association with topical moisturizing creams and occasionally topical steroids. We have also used topical antiseptic treatments such as bathing in a dilute bleach solution or swimming in chlorinated pools to reduce the bacterial burden in the skin. Skin abscesses are a common complication of HIES and may require incision and drainage. Interestingly, after the introduction of prophylactic antibiotics, skin abscesses are unusual. The role of prophylactic antibiotics has not been rigorously investigated in this setting, but there is general consensus in favor of their use to prevent the skin and lung infections. Most physicians cover *S. aureus* with trimethoprim-sulfamethoxazole, a semisynthetic penicillin (e.g., dicloxacillin), or a first-generation cephalosporin. With increasing rates of community-acquired methicillin-resistant *S. aureus* (MRSA) in the community, cultures of eczematoid regions or of the nares can be helpful in selecting a prophylactic antibiotic. If parenchymal lung disease is present, such as bronchiectasis or pneumatoceles, then expansion of antibacterials may be necessary.

The other major recurrent infectious problem in HIES is mucocutaneous candidiasis. This typically manifests as onychomycosis and oral and/or vaginal thrush. *Candida* disseminates typically only when indwelling intravenous catheters are present. Oral antifungals, such as fluconazole, are quite effective in controlling the mucocutaneous candidiasis in HIES and may be used as prophylaxis. Anti-*Aspergillus* antifungals, such as voriconazole, posaconazole, and itraconazole, should be considered for individuals with pneumatoceles as molds are a significant cause of morbidity and mortality for these individuals. Although the overuse of antibiotics and antifungals is currently discouraged in general practice because of concerns about selection for resistant organisms, there is no clear demonstration of increasingly resistant organisms in HIES patients or other patients with primary immunodeficiencies on prolonged prophylactic and suppressive antibiotic therapy; on the contrary, the benefits of this approach are readily appreciated.

One remarkable feature of HIES is the enormous infectious burden with which patients often present. By the time the patient or parent is aware of illness, there is usually nothing subtle about the diagnosis. One of the most difficult problems in the management of HIES is that even in the presence of obvious auscultatory and radiographic evidence of pneumonia, patients do not feel unwell and may be unwilling to undergo invasive diagnostic testing or prolonged courses of therapy. In addition, the hesitance of physicians to believe that patients who appear no different from their baseline are really on the verge of collapse is difficult to overcome. However, aggressive therapy is necessary to try to lessen parenchymal lung damage.

As in most of medicine, the firm establishment of a microbiological diagnosis cannot be overemphasized. Bronchoscopy may help in both obtaining the specimen and mobilizing secretions. Thick inspissated secretions typically plug ectatic bronchi and are tenacious and difficult to clear. High-dose intravenous antibiotics or highly active oral antibiotics may be needed for a prolonged course to eradicate infection and to prevent bronchopleural fistula formation and bronchiectasis. Empirical acute coverage should consider *S. aureus*, *H. influenzae*, and *S. pneumoniae*, and then antibiotics can be tailored to the microbiological findings.

One of the typical features of AD-HIES is that following the resolution of acute pneumonias, pulmonary cysts and bronchiectasis may form. These parenchymal abnormalities then serve as the focus for colonization with *P. aeruginosa*, *Aspergillus* and other fungal species, and nontuberculous mycobacteria. These superinfections are the most difficult aspect of long-term management of HIES. Similar to cystic fibrosis, eradication of *Pseudomonas* and molds is very difficult, but the goal should be to diminish the degree of colonization and disease and treat exacerbations aggressively. The role of surgery to remove infected pneumatoceles and areas of parenchymal abnormalities remains unclear. *Aspergillus* and *Pseudomonas* infections in these areas of parenchymal abnormalities are a major source of morbidity and mortality, but thoracotomy and lung resection appear to have increased complications. The remaining lung may fail to expand normally, and soiling of the pleural space may occur, leading to chronic empyema. Surgery often results in a need for very prolonged chest tube drainage, thoracoplasty, or pneumonectomy, along with intensive parenteral antibiotic treatment. Therefore, pulmonary surgery in HIES should not be undertaken lightly and should be performed at a center with experience with the disease where possible and in most cases after failure of control of infection with antimicrobials.

The role for immune modulators in HIES is unproven. Levamisole was tried in a double-blind, placebo-controlled trial and actually caused an increase in infectious complications in the treatment arm (Donabedian et al., 1982). This is the only published double-blind, placebo-controlled trial of any management intervention in HIES. Immune globulin therapy, either intravenously or subcutaneously, is used for some patients, with anecdotal improvement for at least a subset of patients (Kimata, 1995; Wakim et al., 1998). A blinded study of immunoglobulin replacement in HIES is therefore warranted.

It has been previously supposed that since immunodeficiency is a central part of HIES, the underlying defect in HIES must reside in the leukocytes; thus, bone marrow transplantation has been suggestive as a curative treatment. Cases of bone marrow transplantation in HIES have been reported (Gennery et al., 2000; Nester et al., 1998). The first was a 46-year-old man with HIES who had experienced many bouts of pneumonia from childhood onward. Two of his four children were also affected. He developed a B-cell lymphoma that was treated with a peripheral stem cell transplant from his HLA-identical sister, preceded by total body irradiation. He was maintained on prednisolone and cyclosporine A after the transplant. Serum IgE fell to normal values following transplant and no infections were reported. However, he died 6 months later from interstitial pneumonitis.

The second patient was a 7-year-old girl who was transplanted in an effort to treat her severe HIES. She received marrow from a matched unrelated donor after cytoreductive therapy. Cyclosporine A was used for prophylaxis of graft-versus-host disease. Initially the skin lesions cleared, but 4 years after transplant, she developed recurrent infections, although perhaps more mild, despite full engraftment, and her IgE returned to pretransplant levels. The high IgE is particularly surprising in view of the reported full engraftment of the B-cell compartment, suggesting that the features that led to IgE elevation in this patient may have been somatic, and that the features that made her susceptible to infection may not have been confined to the hematopoietic system.

More recently, successful bone marrow transplants of two unrelated boys with sporadic HIES due to STAT3 mutations have been reported (Goussetis et al., 2010). Both patients had developed high-grade non-Hodgkin's lymphoma in addition to HIES symptoms. After myeloablative conditioning, both received bone marrow from their respective healthy, HLA-identical sibling. Acute or chronic graft-versus-host disease was prevented by the administration of cyclosporine and methotrexate. Initially, severe adenoviral hemorrhagic enteritis occurred, but it ceased after stable engraftment was achieved. Full donor chimerism led to complete immunological correction. Ten and 14 years after transplant, respectively,

serum IgE levels remained normal, no respiratory and skin infections were reported, and even nonimmune manifestations like osteoporosis in one patient resolved. Furthermore, bone marrow transplantation prevented the development of later-occurring complications such as coronary artery aneurysms, brain lesions, or degenerative joint disease.

Now that the genetic etiology of AD-HIES is known, the pathogenesis of both the somatic and immunological manifestations will be delineated. Understanding the various manifestations of this rare disease will help to expand our knowledge of more common disease processes such as staphylococcal susceptibility, eczema, idiopathic scoliosis, and coronary artery disease. In addition, understanding the pathway of STAT3 deficiency will help us to learn the etiology and pathogenesis of related, still poorly understood hyper-IgE syndromes. These advances are expected to lead to further possibilities for treatment.

AR-HIES

In 2004, 13 HIES patients from six consanguineous families with severe recurring infections (pneumonia and abscesses), eczema, high IgE, and eosinophilia were described who fulfilled the criteria for diagnosis of HIES, here designated autosomal recessive (AR)-HIES (Renner et al., 2004). These patients lacked many of the connective tissue and skeletal abnormalities associated with AD-HIES and had increased viral skin infection susceptibility, as well as more neurological symptoms and autoimmune phenomena. A single patient with AR-HIES was reported to have deficiency of the tyrosine kinase-2, or Tyk2 gene, TYK2 (MIM #611521) (Minegeshi et al., 2006). This Tyk2-deficient patient had a unique clinical phenotype. In contrast to other AR-HIES patients, he had Bacillus Calmette-Guérin (BCG) and *Salmonella* infections, which are classically associated with defects of the IL-12/IFN-gamma pathway. However, he did exhibit other clinical features of AR-HIES, including eczema, recurrent sinopulmonary infections, and viral infections. He was found to have a homozygous 4 nucleotide deletion in Tyk2 leading to a premature stop codon (Minegishi et al., 2006). Tyk2 was sequenced in several of the other reported cases of AR-HIES and mutations were not found (Woellner et al., 2010). Recently, a second Tyk2-deficient patient has been described who presented with mycobacterial and cutaneous viral infections but, unlike HIES patients, not with pyogenic bacterial infections (Kreins et al., 2010). This finding suggests that Tyk2 deficiency may predispose to infections with mycobacteria and other intracellular pathogens, while the HIES phenotype may be a less consistent finding that could possibly be explained by other loci that were homozygous in the Japanese patient.

In 2009, mutations in the dedicator of cytokinesis-8 gene (DOCK8) were found to account for the disease of most patients with AR-HIES (Engelhardt et al., 2009; Zhang et al., 2009). DOCK8 deficiency is associated with elevated serum IgE, eosinophilia, eczema, food allergies, sinopulmonary infection, viral skin infections, staphylococcal skin infections, mucocutaneous candidiasis, and a predisposition to malignancy. One of the most distinguishing characteristics between DOCK8 deficiency and AD-HIES is the viral skin infections. Patients with DOCK8 deficiency have had severe chronic therapy-resistant molluscum contagiosum infections, warts, herpes zoster, and recurrent herpes simplex mucosal and skin infections. Malignancies have included squamous cell carcinomas, thought to be related to the human papillomavirus (HPV) infection, and lymphomas. Mortality is frequently at a younger age than with AD-HIES, often occurring in the first and second decades. Connective tissue and skeletal characteristics common in AD-HIES such as retained primary dentition, minimal-trauma fractures, and the characteristic facial appearance are infrequent in DOCK8 deficiency.

Although both AD- and AR-HIES have elevated serum IgE and eosinophilia, there are distinguishing laboratory features. DOCK8 deficiency may be associated with lymphopenia, with most patients having decreased CD4 and CD8 T lymphocytes, B lymphocytes, and natural killer cells, although in other patients lymphocyte subsets have been normal. Another distinguishing feature is diminished serum IgM in DOCK8 deficiency. Serum IgG and IgA levels are variable but frequently normal; specific antibody production has been variable. Lymphocyte proliferation, especially of CD8 T cells, appears abnormal.

DOCK8 is one of 11 members of the DOCK180 family of proteins that are thought to be involved in cytoskeletal rearrangements allowing cell migration, adhesion, and growth. The exact function of DOCK8 remains unknown but is proposed to be involved with cytoskeletal rearrangements important for T-cell activation and effector responses. Most of the reported patients with DOCK8 deficiency have had greatly diminished or absent production of DOCK8 protein. Both homozygous and compound heterozygous mutations have been reported, and large deletions have been frequent.

TREATMENT OF DOCK8 DEFICIENCY

As the genetic etiologies of AR-HIES are elucidated, optimal therapies will be determined. Currently, therapies are largely supportive and similar to those utilized in AD-HIES. As eczema can be severe, involvement of a dermatologist is important to maximize skin care. Treatment of recurrent staphylococcal skin infections is frequently with antiseptic therapies such as dilute bleach baths and chronic antistaphylococcal antibiotics. Prophylactic antimicrobials are also frequently helpful to control sinopulmonary infections. Allergies and asthma are more frequent than in AD-HIES and may require antihistamines as well as inhaled corticosteroids. Although serum IgG is often normal, specific antibodies are variable and immunoglobulin replacement has been used in some of these patients, with anecdotal improvement of the sinopulmonary infections. Improvement of the skin viral infections has not been seen with replacement of immunoglobulin. The widespread molluscum and HPV that are frequently seen are often difficult to treat with standard measures such as salicylic acid, cryotherapy, and imiquimod. There has been some anecdotal evidence with mixed results with interferon-alpha therapy. Hematopoietic stem cell transplantation leading to the cure of DOCK8-deficient patients was reported in 2010.

In the first report, two patients with recurrent respiratory infections, eczema, and disfiguring molluscum contagiosum received transplants from a HLA-matched unrelated donor (Gatz et al., 2010). Patient 1, with additional chronic autoimmune anemia, developed no graft-versus-host disease due to prophylaxis and achieved complete donor cell chimerism until now, 4 years after transplant. His immune functions have normalized, with complete clearance of molluscum contagiosum and absence of bacterial infections. Patient 2, who additionally suffered from severe periodontitis, recurrent skin abscesses, and chronic ulcerative HSV infections, had a more troublesome transplant course. Among others, she developed brain abscesses and an acute Epstein-Barr virus infection despite full donor chimerism. Finally, like patient 1 her immunological functions normalized with complete clearance of molluscum contagiosum and absence of bacterial infections until her last follow-up 2 years after transplant. Both patients now lead a normal and healthy life.

Bittner et al. (2010) described a DOCK8-deficient patient 6 years after transplant. She received bone marrow from her HLA-identical father together with graft-versus-host disease prophylaxis. Only partial myeloablative preconditioning was applied due to severe pulmonary dysfunction. The transplantation was mainly uneventful and led to mixed donor chimerism. Before transplantation she presented with recurrent obstructive bronchitis, severe eczema, candidiasis, molluscum contagiosum infection, and multiple severe food allergies. Now she is alive and well with no findings of eczema and severe infections, decline of IgE levels, and improvement of pulmonary function. However, despite improvement of severity, she still suffers from food allergies.

A further DOCK8-deficient girl with AR-HIES symptoms complicated by Epstein-Barr virus–induced B-lymphoproliferative syndrome, abdominal vasculitis, and celiac trunk aneurysm received a hematopoietic stem cell transplant from her healthy, HLA-identical sister at 9.5 years of age (Barlogis et al., 2010). Total donor chimerism was achieved, together with a decrease of IgE levels and absence of viral infections and vasculitis.

These results suggest that bone marrow transplantation presents an excellent curative treatment option for DOCK8 deficiency, which is otherwise characterized by high morbidity and mortality.

HYPER-IGE OVERLAP SYNDROMES

Interestingly, in rare cases of other defined genetic diseases, the classical triad of HIES with recurrent skin abscesses, recurrent pneumonia, and extreme elevations of IgE is also fulfilled. Antoniades et al. (1996) reported the coexistence of HIES and Dubowitz syndrome, defined by postnatal growth retardation, microcephaly, and characteristic facial appearance. Boeck et al. (1999) reported the coexistence of pentasomy X and HIES. Boeck et al. (2001) further reported the coexistence of HIES and Saethre-Chotzen syndrome (defined by acrocephalosyndactyly, hypertelorism, and ptosis), caused by mutations in the *TWIST* gene. Prolidase deficiency has been associated with hyper-IgE, recurrent lung infections with residual structural abnormalities, and some of the connective abnormalities seen in AD-HIES. Common mechanisms between these syndromes and those of STAT3 and DOCK8 deficiency remain unknown.

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39.

HEPATIC VENO-OCCLUSIVE DISEASE WITH IMMUNODEFICIENCY

Tony Roscioli and Melanie Wong

Veno-occlusive disease with immunodeficiency (VODI) (OMIM235550) is an autosomal recessive primary immunodeficiency disease associated with hepatic sinusoidal obstruction (also known as hepatic veno-occlusive disease [hVOD]) (Roscioli et al., 2006). The onset of VODI usually occurs prior to the age of 6 months. The immunodeficiency comprises severe hypogammaglobulinemia with absent lymph-node germinal centers and tissue plasma cells together with clinical evidence of T-cell immunodeficiency in the presence of normal numbers of circulating T cells. Opportunistic infections, including *Pneumocystis jirovecii*, mucocutaneous candidiasis, and enteroviral or cytomegalovirus infections, occur. The mortality rate for VODI was previously reported as 100% in the first year of life (Roscioli et al., 2006). In contrast, there have been only three deaths among 8 recently ascertained patients older than 1 year, representing a markedly improved prognosis with early recognition and treatment with intravenous immunoglobulin (IVIg) and *Pneumocystis carinii (jirovecii)* pneumonia (PCP) prophylaxis (Cliffe et al., 2012). The gene responsible for VODI, *SP110*, was identified by homozygosity mapping and candidate gene sequencing and represents the first monogenic disorder of a PML nuclear body (PML NB) protein. Targeted sequencing of exons 2, 4, and 5 of *SP110* has detected mutations in 100% of the 13 individuals with VODI evaluated to date (Cliffe et al., 2012; Roscioli et al., 2006).

CLINICAL DESCRIPTION

VODI is a rare disorder originally described in five Lebanese Australian children from three families (Mellis and Bale, 1976). The prevalence of VODI in the Lebanese population in New South Wales has subsequently been estimated to be 1:22,500, implying a carrier frequency of 1:75 (http://www.abs.gov.au/websitedbs/d3310114.nsf/home/Census+data). Two Italian children, one Hispanic child, and one Palestinian child with confirmed cases of VODI have also been identified (Cliffe et al., 2012; Wang et al., 2012; Ganaiem et al., 2013). There has, in addition, been an earlier case report in the Spanish literature of an individual with hVOD and combined immunodeficiency consistent with VODI (Manzanares Lopez-Manzanares et al., 1992).

The clinical features of 20 children with a clinical diagnosis of VODI and a subset of 13 with confirmed *SP110* mutations are summarized in Tables 39.1 and 39.2, respectively (Cliffe et al., 2012; Roscioli et al., 2006). All children in the Australian cohort presented prior to 6 months of age, either with sequelae of immunodeficiency or concurrently with hVOD. The children with VODI who presented without evidence of hepatic involvement were ascertained at a young age because of an affected sibling and were treated early in the disease course with IVIg.

IMMUNODEFICIENCY IN VODI

The immunological features of VODI involve both the B- and T-cell compartments. Serum immunoglobulin levels are low or undetectable, and most VODI patients have reduced circulating T-cell subsets and $CD19^+$ B-cell numbers (Cliffe et al., 2012). Memory B cells are reduced in almost all VODI patients. Further immunophenotyping in two patients with novel mutations (patients I and F in Table 39.2) demonstrated that $CD27^+CD10^-$ B cells were reduced significantly (7.07% ± 1.6%, vs. control 12.8% ± 2.4 %), while $CD27^-CD10^-$(Naïve) B cells were somewhat reduced (63.7% ± 16.2%, vs. control 77.5% ± 0.7%) and $CD27^-CD10^+$ (transitional) B cells were elevated (25.85% ± 15.2%, vs. control

Table 39.1 VODI CLINICAL AND IMMUNOLOGICAL FEATURES

	PHENOTYPE		
CLINICAL FEATURES	PATIENTS FROM SYDNEY WITH VODI (ROSCIOLI ET AL., 2006)	COMMENTS	NEWLY ASCERTAINED PATIENTS WITH VODI (CLIFFE et al., 2012) WITH NOVEL MUTATIONS*
Presenting <6 months	20/20 (100%)		7/8 (patient F > 6 months)
Hepatic failure at initial presentation	4/20 (20%)	1/12 post-HSCT 3/12 no obvious precipitant	0/8
Hepatomegaly at initial presentation	9/20 (45%)	3/6 *P. jirovecii* 2/6 hepatomegaly without hVOD	6/8 1/8 enterovirus & disseminated cytomegalovirus (F)
P. jirovecii infection	12/20 (60%)	7/12 proven; 5/12 suspected	1/8 suspected (F) 1/8 proven (I)
Mucocutaneous candidiasis	2/20 (10%)		1/8
Other features	1/20 (5%)	By age 19 years	1/8 lung fibrosis (H)
Death	19/20 (95%)		3/8 (38%)
Recovery from initial hVOD	4/20 (20%)	1 completely well 1 chronic liver disease requiring hepatic transplantation 1 hVOD post-HSCT 1 developmental disability, chronic aspiration	4/8
Neurological abnormalities	6/20 (35%)	4/7 cerebral infarction 2/7 Leukodystrophy	1/8 Leukodystrophy
Panhypogammaglobulinemia	19/19 (100%)	1/18 loss of normal immunoglobulins at age 4 months	5/5 tested 1/5 low normal levels of IgA and IgM after commencing IVIg
Normal number of lymphocytes	10/11 (92%)		8/8
Normal number of NK cells	12/12 (100%)		3/3
Decreased intracytoplasmic IFN-γ, IL-2, IL-4, IL-10	4/5 (80%)	Low levels at 4 hours, normal/increased levels at 48 hours	1/1 (F)
Decreased number of memory T and B cells	3/4 (75%)		2/3 These cells were present in the individual with the exon 4 duplication (F).

Table modified from Roscioli et al., 2006.

HSCT, hematopoietic stem cell transplantation; IVIg, intravenous immunoglobulin; hVOD, hepatic veno-occlusive disease.

* Patients I, F, 2, and 3 in Table 39.2: I = c.78_79delinsAT (p.Ile27Leu), F = c.319_325dup (p.Ser109TrpfsX5), 2 = c.667 + 1dup, 3 = c.373delA (p.Th r125LeufsX3)

8.49% ± 3.4%). Patients also showed a marked reduction in $CD27^{+}IgD^{-}$ (switched memory) B cells and an absence of IgG^{+} or IgA^{+} B cells. Histological studies have shown absence of both lymph-node germinal centers and tissue plasma cells (Roscioli et al., 2006). Immunocytochemistry of CD40L stimulated B cells in a representative sample of VODI patients demonstrated that their cells were unable to isotype switch to IgG or IgA. Furthermore, compared with normal controls, total B cells as well as naïve B cells from VODI patients, when activated in vitro with CD40L +/- IL-21, showed marked reduction in immunoglobulin production (IgM was 6-fold reduced; IgG and IgA were >100-fold reduced), demonstrating that *SP110* mutations compromise the intrinsic ability of B cells to differentiate into immunoglobulin-secreting cells and to undergo isotype switching in response to T-dependent stimuli (Cliffe et al., 2012).

VODI patients show a predisposition to *P. jirovecii* and mucocutaneous candidiasis, consistent with impaired T-cell immunity. While the numbers and percentages of circulating T-cell subsets are moderately decreased and T-cell proliferation assays are normal in VODI patients, the $CD45RO^{+}CD27^{-}$ (memory) T cells are almost absent, with resultant predominance of naïve T cells. Intracytoplasmic cytokine expression after stimulation with phorbol myristate acetate and ionomycin is significantly reduced: IFN-γ 1 to 4% (reference range 25–30v); IL2 1 to 3% (12–32%); IL4 1 to 2% (4–7%), and IL10 1 to 2% (1–6%). NK activity was normal in the seven patients with *SP110* mutations tested.

HEPATIC DISEASE IN VODI

Hepatic disease in VODI is characterized by hepatomegaly or hepatic failure. Ninety percent of the children with VODI

Table 39.2 CLINICAL FEATURES OF INDIVIDUALS HOMOZYGOUS FOR *SP110* MUTATIONS

PATIENT	SP110 MUTATION	PRESENTATION	SERUM Ig's	MEMORY T/B CELLS	T CELL CYTOKINES	CLINICAL FINDINGS	DECEASED?
AII.1[1] Lebanese	c.642delC exon 5	Age 5 months: immunodeficiency, thrombocytopenia, hVOD	↓	N/A	N/A	Left hemiparesis[3], recurrent hVOD with GVHD post-HSCT	Yes
BII.1[1] Lebanese		Age 7 months: immunodeficiency	↓	N/A	N/A	Chronic lung disease secondary to recurrent aspiration	Yes (age 19 years)
BII.2[1] Lebanese		Age 6 months: hepatosplenomegaly, ascites, hVOD	↓	↓	↓	Well	No
CII.1[1] Lebanese		Age 4 months: hepatosplenomegaly, ascites, hVOD, thrombocytopenia, mucocutaneous candidiasis	↓	↓	↓	Chronic liver disease, portal hypertension post-hepatic transplantation	Yes
DII.1[1] Lebanese		Age 3 months: hepatosplenomegaly, ascites, hVOD	↓[4]	↓	↓	Hemophagocytic syndrome post-hepatic transplantation	Yes
G[1] Lebanese		Age 3 months: hepatosplenomegaly, ascites, hVOD	↓	↓	↓	Pulmonary hemorrhage, multi-organ failure	Yes
J[1] Lebanese		Age 3 months, respiratory distress	↓	↓	N/A	SIADH, idiopathic cerebrospinal leukodystrophy	No
6[2] Lebanese		Age 3 months: chronic cough, diarrhea hepatosplenomegaly 8 years, hVOD > 12 years	↓	N/A	N/A	Idiopathic left frontal lobe calcified cyst, Epilepsy, CMV colitis, post-diarrheal encephalomyelitis with lower limb paralysis, cerebrospinal leukodystrophy, oesophageal candidiasis, duodenal lymphocytic infiltrate	No
7[2] Lebanese	c.642delC presumed	Age 2 months: chronic diarrhea, failure to thrive, middle ear and respiratory infections, hepatosplenomegaly, thrombocytopenia	N/A	N/A	N/A	Microcephaly, Hepatic biopsy consistent with hVOD	Yes, 11 months diarrhea leading to septic shock
8[2] Lebanese	c.642delC presumed	Age 5 months: upper respiratory illness, age 8 months chronic diarrhea, hepatomegaly, thrombocytopenia	N/A	N/A	N/A	Hepatic biopsy consistent with hVOD	Yes, 3.5years, diarrhea leading to septic shock
9[2] Lebanese	c.642delC presumed	Age 2 months: ascites, hepatomegaly, anaemia, thrombocytopenia	N/A	N/A	N/A	Hepatic biopsy consistent with hVOD	Yes, 2.5 months otitis, diarrhea, pneumonia
EI.1[1] Lebanese	c.40delC in exon 2	Age 3 months: immunodeficiency, thrombocytopenia, hepatosplenomegaly without definite evidence of hVOD	↓	N/A	N/A	Enteroviral and *P. jiroveci* infection	Yes

(continued)

Table 39.2 (CONTINUED)

PATIENT	SP110 MUTATION	PRESENTATION	SERUM Ig's	MEMORY T/B CELLS	T CELL CYTOKINES	CLINICAL FINDINGS	DECEASED?
I[2] Hispanic	c.78_79delinsAT (p.Ile27Leu) exon 2	Age 3 months	↓	↓	↓	Stable and well	No
F[2] Italian	c.319_325dup GGTGCTT exon 4	Age 11 months: hepatosplenomegaly, disseminated cytomegalovirus infection, rotavirus gastroenteritis, vulvar abscesses, hVOD	↓initially	↓	N/A	Recovering from hVOD, well	No
2[2] Italian	c.667+1dup exon 5 splice site	Age 3 months: hepatosplenomegaly, failure to thrive, respiratory distress/lung fibrosis, diarrhea	↓	↓	N/A	Hepatic biopsy consistent with sinusoidal dilatation, moderate central vein and perivenular subsinusoidal fibrosis; stable with improvement	No
3[2] Palestinian Arabic	c.373del exon 4	Age 3 months: diagnosis of VODI confirmed with cascade testing prior to illness onset. No hepatomegaly or liver function abnormalities	N/A	N/A	N/A	Stable and well	No

Modified from Roscioli et al. (2006).

Although families A, B, and C are not known to be related, they are believed to have a common ancestor. Individuals AII.1, BII.1, BII.2, CII.1, G, and J were included in the initial homozygosity mapping analysis.

GVHD, graft-versus-host disease, HSCT, hematopoietic stem cell transplantation; hVOD, sinusoidal obstruction syndrome; SIADH, syndrome of inappropriate antidiuretic hormone secretion.

[1]Reported in Roscioli et al. (2006).

[2]Reported in Cliffe et al. (2012).

[3]Secondary to cerebral white matter abnormality.

[4]IgA and IgM serum concentrations increased to lower limit of normal while on IVIG.

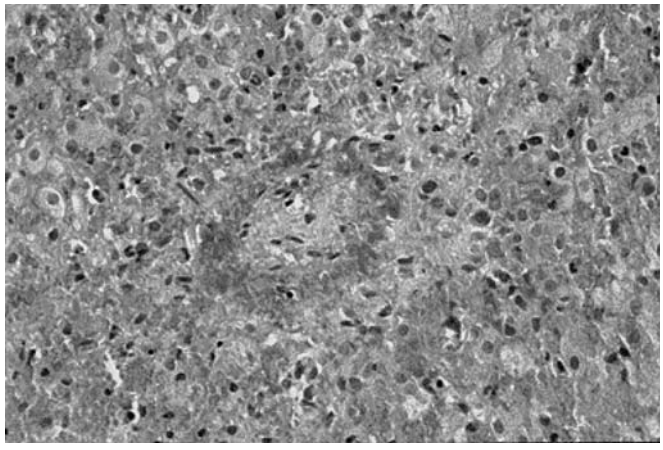

Figure 39.1 Hepatic biopsy showing vascular obliteration, perivenular fibrosis, zone 3 fibrosis, and hepatocyte dropout from a girl who presented at age 5 months with hepatomegaly and ascites (Picro-Mallory stain, ×100).

have presented either with hepatomegaly (83% with preceding infection) or hepatic failure (53% with preceding infection). A significant improvement in the hVOD recovery rate from 20% in the initially described cohort to 100% in the recently identified group has been observed. A typical hepatic biopsy from an affected individual that demonstrates the features of vascular obliteration, perivenular fibrosis, zone 3 fibrosis, and hepatocyte dropout that are diagnostic of hVOD is shown in Figure 39.1. It is currently unknown whether hVOD is a direct manifestation of *SP110* mutations or whether it is related to altered apoptosis in hepatic sinusoids or secondary to infection. It is unusual for primary or acquired immunodeficiencies to be associated with hVOD, and there are only isolated reports of such associations (Buckley and Hutchins, 1995; Washington et al., 1993). However, in children with VODI, hVOD appears to develop after infection. In hematopoietic stem cell transplantation (HSCT) the presence of hVOD prior to day 20 and before

engraftment also suggests that T- or B-cell–mediated mechanisms are not the primary cause of hVOD.

OTHER CLINICAL FEATURES

Thirty percent of children with VODI had neurological involvement. Four unrelated affected children had multiorgan failure associated with extensive cerebral necrosis on postmortem examination. A striking finding is the presence of cerebrospinal leukodystrophy in 3 (20%) VODI patients. Patient 5 (Table 39.2) had a leukodystrophy of unknown etiology and patient 6 developed this complication after a CMV-related gastroenteritis. In patient AII.1, the initial diagnosis of a cerebrovascular accident with a right-sided cerebral white matter lesion, presumed to be *Toxoplasma* or a porencephalic cyst was revised to being more consistent with cerebrospinal leukodystrophy. Child J developed a syndrome of inappropriate antidiuretic hormone secretion (SIADH) and an idiopathic cerebrospinal leukodystrophy, resulting in upper motor neuron lesions affecting the upper limbs and swallowing, which improved slowly. Lung fibrosis of unknown etiology was identified only in patient 2 (*SP110* c.667+1dupG).

OUTCOMES

VODI is associated with 100% mortality in the first year if unrecognized and untreated with IVIg and PCP prophylaxis. Improved recognition and early management have reduced mortality (Cliffe et al., 2012). Should hVOD recovery occur, recurrence appears to be prevented by continuation of IVIg and PCP prophylaxis. One child (AII.1, Table 39.2) died following recurrence of hVOD after HSCT at age 6 years. Chronic hepatic disease of unknown etiology resulting in hepatic failure occurred in one child in her teenage years after an disease-free period (CII.1, Table 39.2). She underwent a hepatic transplant but died as a result of multiple complications.

No significant difference in the clinical manifestations of VODI has been observed between individuals with *SP110* exon 2, exon 4, and exon 5 mutations. One individual with an exon 4 insertion/duplication had later onset of disease at 11 months, disseminated cytomegalovirus (CMV) infection, and normal numbers of memory T and B cells and intracellular cytokine production, differentiating her from the rest of the cohort.

DIFFERENTIAL DIAGNOSIS

The primary differential diagnosis for hVOD is environmental alkaloid or sinusoidal cell toxicity. However, hVOD has also been reported in association with alcoholic cirrhosis (Kishi et al., 1999), ataxia-telangiectasia (Srisirirojanakorn et al., 1999), osteopetrosis (Corbacioglu et al., 2006), and hypereosinophilic syndrome. hVOD associated with severe combined immunodeficiency (SCID) has been described (Washington et al., 1993) and was observed in a postmortem HIV cohort (Buckley and Hutchins, 1995). The lack of a strong association between immunodeficiencies and hVOD suggests that hVOD is a primary and specific feature of VODI.

MANAGEMENT

EVALUATIONS AT INITIAL DIAGNOSIS

Full blood count and biochemical assessment of hepatic function should be undertaken. A clinical assessment for portal hypertension, a coagulation profile, and a hepatic Doppler ultrasound examination are extremely helpful for identifying the presence of intrahepatic venous obstruction and should be undertaken prior to consideration of hepatic biopsy for the histological diagnosis of hVOD. Evidence of impaired clotting (if unable to be corrected) and/or significant portal hypertension are contraindications to hepatic biopsy.

The initial immune evaluation should include serum immunoglobulin levels, T- and B-cell numbers and percentages, and T-cell proliferative response to mitogen. Extensive immune testing for memory B- and T-cell numbers and intracellular cytokine responses to phorbol ester stimulation may be of interest. *SP110* molecular genetic testing should be done to confirm the clinical diagnosis and to assist with the genetic counseling process. Sequencing of the 19 *SP110* coding exons should be performed if no mutations are identified in exons 2, 4, and 5.

TREATMENT OF SYMPTOMS

IVIg administration and cotrimoxazole prophylaxis for PCP should commence at diagnosis, or in presymptomatic siblings after the confirmation of homozygous *SP110* mutations. Specific infections should be treated appropriately. Some evidence suggests that early treatment may reduce the risk of hVOD. It is unknown whether hVOD resolution and/or prevention with IVIg is due to an immunomodulatory role of IVIg or results from a decrease of infections.

SURVEILLANCE

Surveillance of hepatic function, platelet count, and hemoglobin in children with VODI should be undertaken on a regular basis as hepatic failure and portal hypertension may occur. In addition, routine monitoring of complete blood count and renal and hepatic function and periodic assessment of trough IgG levels are recommended. Pulmonary function testing may be indicated to monitor for the sequelae of significant lower respiratory tract infection. Continuing awareness and appropriate tests for the investigation of possible infection are essential, as in other patients with a significant combined immunodeficiency.

Agents known to predispose to hVOD such as cyclophosphamide and senecio alkaloids/bush teas should be avoided in VODI. hVOD has been reported in the Australian cohort of

VODI patients following HSCT, so individuals with VODI were thought to have, at very minimum, the population risk of hVOD after HSCT, suggesting that this form of transplantation was contraindicated. Recently however, HSCT has been reported as a successful therapy for VODI in 3 out of 5 children with a specific mutation in *SP110* confirming that modified conditioning regimes pre-transplantation increase the chance of a successful outcome (Ganaiem et al., 2013). Hepatic transplantation may also have an increased risk of complications. One child with VODI (DII.1) is known to have developed hemophagocytic syndrome after hepatic transplantation. The safety of hepatic transplantation in VODI is not clear as it appears to have a high rate of complications in the VODI cohort studied to date.

GENETIC COUNSELING

VODI is inherited in an autosomal recessive manner, and parents of an affected child are asymptomatic obligate heterozygotes. The recurrence risk for siblings of an affected child is 25%. Carrier testing for at-risk relatives and prenatal (or preimplantation) genetic diagnosis for pregnancies at increased risk after genetic counseling are possible if both disease-causing mutations in a family are identified. Molecular genetic testing of siblings of a proband who are younger than age 12 months to allow early diagnosis and treatment should be discussed with the family. VODI immunodeficiency penetrance has been 100% in individuals with mutations in *SP110*, although it is possible that this risk is inflated due to ascertainment bias. Similarly, hVOD has been described in all probands or their affected siblings.

SP110 AND MOLECULAR GENETICS

The Sp110 nuclear body protein is a member of the Sp100/Sp140 PML NB protein family. The PML NB is a nuclear macromolecular complex deployed to areas of active host/viral DNA replication, transcription, and repair and has been reported to be involved in apoptosis, cell-cycle control, and the immune response (Hofmann and Will, 2003; Kiesslich et al., 2002; Muratani et al., 2002; Negorev and Maul, 2001; Rivera et al., 2003). Sp110 contains a Sp100 domain involved in dimerization with other Sp100 family proteins (amino acids [a.a.] 6–159), a nuclear localization signal (a.a. 288–306), and a nuclear hormone interaction domain (LXXLL type), which may act as an all-trans retinoic acid (ATRA) response element. Other domains facilitating chromatin-mediated gene transcription include a SAND domain (a.a. 452–532), a plant homeobox domain (a.a. 537–577), and a bromodomain (a.a. 606–674) (Bloch et al., 2000). Epstein-Barr virus (EBV)-transformed B cells from an individual with VODI have shown an absence of nuclear Sp110 expression but with maintenance of normal numbers of PML NBs, consistent with *SP110* having a role in the immune response without being essential for PML NB assembly (Roscioli et al., 2006).

Further evidence for the involvement of the PML NB and Sp100 in the immune response include their induction by IFN-γ (Chelbi-Alix et al., 1995; Guldner et al., 1992; Lavau et al., 1995; Stadler et al., 1995), inactivation by HSV-1 (Chelbi-Alix and de The, 1999; Negorev and Maul, 2001), the disruption of PML-NBs by the CMV early proteins IE1 and 2 (Rio et al., 1994), the EBV immediate-early protein BZLF1 (Adamson and Kenney, 2001), and a possible PML anti-Ebola virus and anti-rabies virus effect (Bjorndal et al., 2003; Blondel et al., 2002). The hepatitis C virus (HCV) core protein has been shown to interact with the Sp110b HCV core binding domain (a.a. 389–453), causing its inactivation and sequestration from the nucleus, resulting in ATRA-induced cell death (Watashi et al., 2003). These studies and the VODI phenotype are consistent with an *SP110* role in infectious disease resistance.

SP110 is expressed primarily in leukocytes and the spleen; it is induced by IFN-γ and ATRA. Expression microarray experiments have demonstrated altered levels of proteins affecting late B-cell differentiation pathways including decreased CD27 expression, down-regulation of the TNFR superfamily and SLAM pathways (SLAMF3/CD229 and SLAMF5/CD84) consistent with of the failure of production of memory B cells (Cliffe et al., 2012). The Sp110 nuclear body protein has three major isoforms: (1) NM_004509 (78.438 kd; lacking exon 17); (2) NM_004510 (61.940 kd; includes an alternate exon 15 and terminates within exon 15); and (3) NM_080424 (81.211 kd; includes exon 17 and terminating at exon 19). The Sp110b protein isoform has been described as showing activity as a potent transcriptional co-repressor of retinoic acid receptor alpha (RARα), perhaps via competitive exclusion of activators at receptor (Watashi et al., 2003).

PATHOLOGICAL ALLELIC VARIANTS

All reported mutations cluster within the first part of the gene (exons 2, 4, and 5), and so are predicted to affect all isoforms of the Sp110 mRNA. The majority of pathogenic mutations cause a frameshift with consequent protein truncation. The one exception to date is the c.78_79CA>AT mutation. This dinucleotide substitution mutation includes the silent third base of codon 26 (GCC>GCA, both of which encode Alanine) and the adjacent first base of codon 27 (ATA>TTA Isoleucine to Leucine). This predicted substitution is relatively conservative and ordinarily well tolerated by proteins; however, in this instance, the mutation is located within the highly conserved Sp140-Sp110 dimerization domain. A multispecies alignment of the protein sequence in this region shows that Isoleucine27 is almost absolutely conserved, suggesting that this residue may have a significant functional role in protein–protein interactions and may mediate the Sp140-related recruitment of Sp110 into the nuclear body. The dinucleotide deletion/insertion mutation has been shown to produce profound effects on Sp110 protein stability (Cliffe et al., 2012). The key pathogenic event in the development of VODI in all cases reported to date therefore appears to be of the lack of fully functional Sp110 protein.

OTHER *SP110* VARIANTS

No other Mendelian disorders for *SP110* or contiguous gene deletion disorders including the *SP110* region have been described. Tosh et al (2006) reported transmission disequilibrium for alleles of *SP110* in *Mycobacterium tuberculosis* infection in individuals of West African heritage. However, a replication study in a similar population did not identify the same association (Thye et al., 2006). A well-designed study by Szeszko et al. (2007) failed to detect a significant association between alleles of *SP110* and *M. tuberculosis* infection in Russian Caucasians. It is notable that the three studies cited compare Mantoux-positive and Mantoux-negative individuals rather than disease progression in individuals known to be exposed to *M. tuberculosis*.

Previous case-controlled studies using single-nucleotide polymorphisms (SNPs) have also reported associations between HSCT-induced hVOD and SNPs in the carbamyl phosphate synthetase 1 (*CPS1*), factor V Leiden (*FVL*), *HFE*, and glutathione S-transferase genes (*GSTM1* and *GSTT1*), with relative risks of 8.6 for the homozygous HFE C282Y allele and 4.12 for the *GSTM1* null allele (Kallianpur, 2005; Kallianpur et al., 2005; Srivastava et al., 2004). No independent replication of these findings has been performed. There has been no report of *SP110* mutations in individuals described to have hVOD alone.

SUMMARY

VODI is due to mutations in the *SP110* gene and is the first immunodeficiency to be associated with the PML NB. Although rare globally, it shows higher frequencies in some population isolates and has been reported in populations with Mediterranean ancestral admixture both in Europe and North America as well as Hispanic populations. The clinical pattern and lack of genetic heterogeneity thus far make VODI an important and specific differential diagnosis for immunodeficiency syndromes.

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40.

WHIM SYNDROME

George A. Diaz

CLINICAL FEATURES

The term "WHIM syndrome" was coined to describe a rare combined immunodeficiency disease characterized by the clinical features of *W*arts, *H*ypogammaglobulinemia, *I*mmunodeficiency, and *M*yelokathexis (Wetzler et al., 1990; Fig. 40.1). The latter manifestation is a form of peripheral neutropenia in the face of hypercellularity of mature neutrophils in the bone marrow (Zuelzer, 1964). The features described by Wetzler et al. in two sisters and, by history, in their deceased father included noncyclical neutropenia with transient elevation of neutrophil counts during infectious episodes, recurrent bacterial infections (otitis media, pneumonia, cellulitis, urinary tract infection), and extensive dermal and/or cervical warts with no specific human papillomaviral isotype susceptibility. Immunological investigation revealed depression of all immunoglobulin subtypes and profound lymphopenia, particularly of B cells. As additional cases have been ascertained, it has become apparent that the clinical expression of the disorder can be quite variable. The spectrum of disease manifestations, genetics, and disease pathophysiology will be reviewed in the following sections.

HEMATOLOGIC FEATURES

Neutropenia is marked in WHIM syndrome, with absolute neutrophil counts generally significantly below 1,000/mm^3. In contrast to neutropenic states caused by mutations in the *CSF3R* (Dong et al., 1994), *ELANE* (Dale et al., 2000; Horwitz et al., 1999), or *WAS* (Devriendt et al., 2001) genes, in which normal development of the myeloid lineage is arrested, neutrophil maturation is not defective in WHIM syndrome. Indeed, bone marrow samples are remarkable for hypercellularity of mature myeloid cells, but these often display morphological changes suggestive of apoptosis (nuclear hypersegmentation, pyknosis, cytoplasmic vacuolation). Vacuolation of eosinophils has also been described in a subset of cases (Gorlin et al., 2000; Latger-Cannard et al., 2006). Other less frequently reported quantitative abnormalities including deficits in peripheral blood monocytes (Balabanian et al., 2005; Siedlar et al., 2008) and increased natural kille (NK) cell counts (Wetzler et al., 1990) in a subset of patients.

An important diagnostic feature of the neutropenia of WHIM syndrome is the capacity of patients to transiently elevate their peripheral neutrophil counts in the setting of infection. These mobilized neutrophils appear to be functionally normal as assessed by tests of neutrophil migration, opsonization, and phagocytosis; functional deficits have been observed in patients manifesting isolated myelokathexis (Plebani et al., 1988; Weston et al., 1991). Myelokathexis is a genetically heterogeneous condition (see below), so these cases are not likely to represent *bone fide* WHIM cases. In addition to infection, neutrophil mobilization can also be triggered by epinephrine, glucocorticoids, and challenge with tetanus toxoid (Hord et al., 1997; Mentzer et al., 1977). The cytokines granulocyte- and granulocyte/monocyte-colony stimulating factor (G-, GM-CSF) have been used therapeutically to elevate neutrophil counts in individuals affected with isolated myelokathexis or WHIM syndrome (Bohinjec and Andoljsek, 1992; Cernelc et al., 2000; Hess et al., 1992; Weston et al., 1991; Wetzler et al., 1992).

In contrast to the myeloid hyperplasia, lymphocyte abundance is normal in bone marrow samples from WHIM syndrome patients. However, peripheral lymphopenia is a fairly consistent disease feature (Arai et al., 2000; Goddard et al., 1994; Gorlin et al., 2000; Hess et al., 1992). In particular, profound B lymphopenia is uniformly present, while

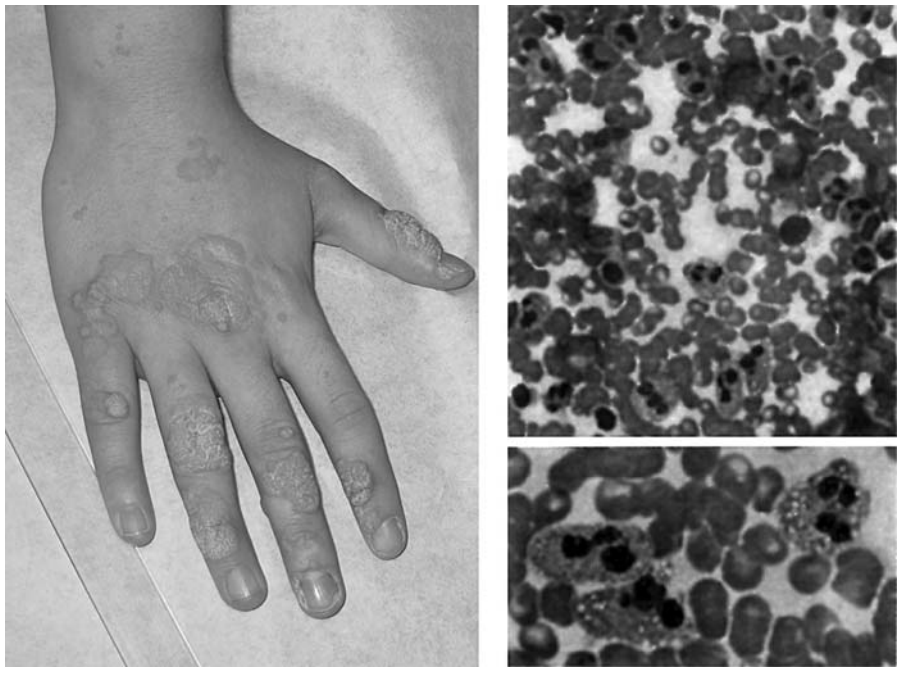

Figure 40.1 *Characteristic findings in WHIM syndrome.* The left panel shows an example of extensive and treatment-refractory HPV verrucosis on the hand of a young patient. The panels at right show the characteristic myeloid hyperplasia (*top*) and bizarre, hypersegmented nuclei and cytoplasmic vacuolation in a bone marrow aspirate sample from the original description characterizing the syndrome. (Panels at right adapted from Wetzler et al, 1990.) (See Color Plate).

T lymphopenia is present in some cases (Arai et al., 2000; Gorlin et al., 2000; Hess et al., 1992) but absent in others (Hord et al., 1997; Mentzer et al., 1977; Wetzler et al., 1990). Even when T lymphopenia is present, the CD4/CD8 ratio is generally been normal (Goddard et al., 1994; Taniuchi et al., 1999; Wetzler et al., 1990).

IMMUNOLOGICAL FEATURES

Even though hypogammaglobulinemia is included in the acronym defining the syndrome, this feature is relatively variable. Although immunoglobulin deficiencies have been reported in most cases in which levels were assessed, serial measurements have documented variation ranging from moderate deficiency to essentially normal levels (Arai et al., 2000; Christ and Dillon, 1997; Gorlin et al., 2000; Imashuku et al., 2002; Mentzer et al., 1977). Isolated IgG deficiency is most frequent, but additional deficiencies of IgM (Hord et al., 1997) or IgA and IgM (Wetzler et al., 1990) have been reported.

To date, little has been published about the effects of the disease on lymphoid organs. An inguinal lymph node biopsy performed after ipsilateral challenge with diphtheria–tetanus toxoid vaccine in a father and daughter with the full WHIM phenotype showed absent or hypoplastic lymphoid follicles and diminished numbers of plasma cells (Mentzer et al., 1977), although the genetic basis of the disease in this family has not been confirmed. Abdominal ultrasonography has demonstrated mild splenomegaly in several patients (Siedlar et al., 2008; Wetzler et al., 1990), but no histological data have been published to provide insight into the nature of the disease manifestations at the level of lymphoid organ organization. Of note, specific deficits in switched memory B cells ($CD27^+$, IgD^-) has been described in WHIM patients, consistent with disturbed B-cell maturation (Gulino et al., 2004).

Less information is available with regard to T-cell function, but abnormalities have been described in a number of patients. Tests of cell-mediated immune function by intradermal antigen challenge have been normal in most patients in which results were reported (Arai et al., 2000; Hord et al., 1997; Mentzer et al., 1977) but were impaired in some (Mentzer et al., 1977; Wetzler et al., 1990). In the same patients with a paucity of switched memory B cells, an increase was found in the number of memory effector T cells, particularly $CD8^+$ cells, but this increase was found to be associated with oligoclonal expansion of T-cell populations (Gulino et al., 2004).

DISEASE COMPLICATIONS

Although the neutropenia and lymphopenia present in most WHIM patients can be profound, the infectious complications of the disease can be relatively mild, as the reactive leukocytosis permits a good response to antibiotic therapy and resolution of infection. A significant proportion of reported cases have not been diagnosed until adulthood, and only a single case of mortality secondary to infection has been reported (Wetzler et al., 1992). However, most patients report recurrent bacterial infections from early childhood ranging from recurrent otitis media, sinusitis, and urinary tract infection at the mild end of the spectrum to pneumonia, thrombophlebitis, omphalitis, osteomyelitis, cellulitis, and abscesses at

the more severe end. Susceptibility to bacterial pathogens is not restricted, and infections have been reported with *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Proteus mirabilis* species. Recurrent pneumonias may in some cases lead to severe bronchiectasis.

Despite some degree of T-cell lymphopenia or dysfunction in most WHIM patients, immunity to viral pathogens is generally robust. Live viral vaccines are well tolerated with no reported adverse outcomes, and even anergic individuals with low T-cell counts are not susceptible to opportunistic viral infections. The principal exception is a susceptibility to human papillomavirus (HPV). Onset of HPV infections can be from infancy to adolescence, with manifestations ranging from relatively few and scattered warts to extensive cutaneous and genital papillomatosis (Gorlin et al., 2000). Condyloma acuminata in affected females can be severe enough to require laser ablation and vulvectomy secondary to dysplastic changes (Wetzler et al., 1990). Of note, disseminated verrucosis is generally persistent and refractory to treatment with ablation or immunomodulatory therapies but can spontaneously resolve for prolonged periods (Hagan and Nguyen, 2007; Tarzi et al., 2005). A second family of viral pathogens that may cause more severe disease in WHIM patients are the herpesviruses, as several reports have documented severe recurrent herpes infections (Aprikyan et al., 2000; Balabanian et al., 2005; Goddard et al., 1994; Imashuku et al., 2002; Tarzi et al., 2005).

With the description of more cases, it has become apparent that viral-associated neoplastic disease represents a major clinical problem for WHIM patients. Three cases of early-onset B-cell lymphoma following Epstein-Barr virus (EBV) infection have been reported. In one report, an excellent response to chemotherapy was obtained (Chae et al., 2001), but the outcome was fatal in the two other cases (Imashuku et al., 2002; Tassone et al., 2009). In addition to EBV-associated cancer, mortality secondary to an HPV-associated vulval cancer has also been reported (Vinurel et al., 2008). Non–EBV-associated cancers described in WHIM patients include a fatal brain tumor (Montelibano et al., 2007) and a case of Kaposi sarcoma (Diaz, 2005). This latter tumor is associated with human herpesvirus 8, further reinforcing the suggestion that WHIM patients may have an additional susceptibility to herpesviruses.

Apart from infectious and neoplastic sequelae, other disease associations in WHIM syndrome include complex congenital heart disease in 2 patients out of approximately 50 reported, a significantly higher incidence than expected (Gulino et al., 2004; Taniuchi et al., 1999), and hypothyroidism and insulin-dependent diabetes in one patient (Takaya et al., 2009). Idiopathic mental retardation was described in another patient (Tassone et al., 2009). As discussed below, the mutant gene in these cases, *CXCR4*, plays an important role in the biology of cardiovascular and brain patterning and in normal immune function, raising the possibility that these developmental and autoimmune phenomena are a manifestation of abnormal CXCR4 receptor signaling.

Table 40.1 lists the clinical characteristics of a collection of WHIM syndrome patients with confirmed *CXCR4* mutations.

GENETIC STUDIES

By using familial WHIM cases with apparently dominant inheritance for genetic studies, the disease locus was linkage mapped to chromosome 2q21 and mutations were detected in the chemokine receptor gene *CXCR4*, thus identifying the molecular basis of the disease (Hernandez et al., 2003). *CXCR4* encodes a 352-amino acid G-protein–coupled receptor that binds to the chemokine CXCL12. The disease-associated mutations cluster very tightly at the terminus of the CXCR4 cytoplasmic tail domain. All mutations described to date are either nonsense or small deletions that truncate or frameshift the receptor at a point between 19 and 10 residues from the normally encoded stop codon, with one mutation generating a nonsense peptide that extends beyond the in-frame termination codon (Fig. 40.2). Recurrence has been documented for three mutations, R334X, S338X, and S339fs342X, with the former representing the preponderance of novel occurrences (11/21 unique families or individuals). The clinical features in the patients with the minimal 10-residue truncation, R343X (Hernandez et al., 2003; Hord et al., 1997), were not markedly different than those seen in patients with the more extensive 19-residue E334X truncation (Gorlin et al., 2000; Wetzler et al., 1990), suggesting that there is no strong genotype–phenotype correlation based on the extent of the truncation.

The clinical variability observed in WHIM syndrome, particularly within families known to carry mutant alleles, likely reflects the effects of modifier genes, but genetic heterogeneity may also contribute to the broad phenotypic spectrum. *CXCR4* mutations were excluded in a pedigree in which myelokathexis was inherited as an apparent autosomal recessive trait that was not associated with hypogammaglobulinemia or warts (Bohinjec, 1981; Hernandez et al., 2003). The existence of WHIM patients expressing the complete disease spectrum, but in whom *CXCR4* was wild type, confirmed genetic heterogeneity for the disease (Balabanian et al., 2005). Deficient transcriptional expression of the G-protein–coupled receptor kinase *GRK3*, a negative regulator of CXCR4 signaling, was demonstrated in one of these patients, representing the first non-*CXCR4*-mediated case of WHIM syndrome to be characterized at a molecular level (Balabanian et al., 2008). Mutations in additional genes involved in *CXCR4* signaling are likely be identified in other phenocopy cases in the future.

Table 40.2 lists the frequency of different genetic etiologies causing WHIM syndrome.

CXCR4 FUNCTION AND DISEASE PATHOPHYSIOLOGY

CXCR4 is a chemokine receptor that is important in hematopoietic and extrahematopoietic development and has a well-studied role as a co-receptor for HIV-1 (Feng et al., 1996). Like all chemokine receptors, CXCR4 has a conserved heptahelical transmembrane structure typical of G-protein–coupled receptors (GPCR) and mediates signal transduction via multiple pathways. In contrast to chemokines secreted in response to infection/inflammation, CXCR4 is considered a homeostatic chemokine that is expressed constitutively

Table 40.1 CLINICAL CHARACTERISTICS OF A COLLECTION OF WHIM SYNDROME PATIENTS WITH CONFIRMED *CXCR4* MUTATIONS

ANC	IGG DEF.	B	T	ANERGY	HPV	OTHER
+	+	+	-	-	+	Fatal meningitis
+	+	+	-	+	+	Fatal brain tumor
+	+	+	+	+	+	
+	+	-	-	-	+	
+	+	?	?	?	-	
+	?	?	?	?	+	B-cell lymphoma
+	+	+	+	?	+	
+	+	+	+	?	+	
+	+	+	+	?	+	
+	+	+	+	?	+	
+	+	?	?	?	+	
+	+	-	-	?	+	Hypothyroidism, insulin-dependent diabetes mellitus
+	+	-	-	?	-	Congenital heart disease
+	+	+	+	?	+	
+	+	+	+	?	-	
+	+	+	+	-	+	Kaposi sarcoma
+	+	+	+	-	+	
+	+	+	+	?	+	Bronchiectasis
+	?	?	?	?	+	Fatal vulvar cancer
?	?	?	?	?	+	Patient death by 40 yrs.
+	+	?	?	?	-	Cause unknown
+	+	+	+	+	+	
+	+	+	+	+	-	
+	+	+	+	+	-	
+	-	+	+	?	+	Fatal B-cell lymphoma
+	-	+	+	?	+	
+	+	+	+	-	+	Congenital heart disease
+	-	+	+	+	+	Recurrent herpes infection
+	-	+	+	?	-	
+	-	+	+	?	+	
+	+	+	+	?	-	Mental retardation
+	+	-	-	?	+	
+	?	+	+	?	+	
+	+	+	+	?	+	
+	+	+	+	-	-	
+	+	?	?	?	+	

in specific lymphoid compartments (Moser and Loetscher, 2001). The receptor has a single known cognate ligand, CXCL12 (formerly SDF-1). Both CXCR4 and CXCL12 are expressed widely in hematopoietic and nonhematopoietic tissues, including heart, vascular endothelium, and brain. Consistent with this expression pattern, homozygous null *Cxcr4-/-* mice displayed embryonic lethality with cardiac, cerebellar, and vascular malformations and deficits in neutrophil and B-cell production (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998).

CXCR4 SIGNALING

CXCR4-mediated signaling involves several different pathways. These include the canonical Ga_i-coupled G-protein

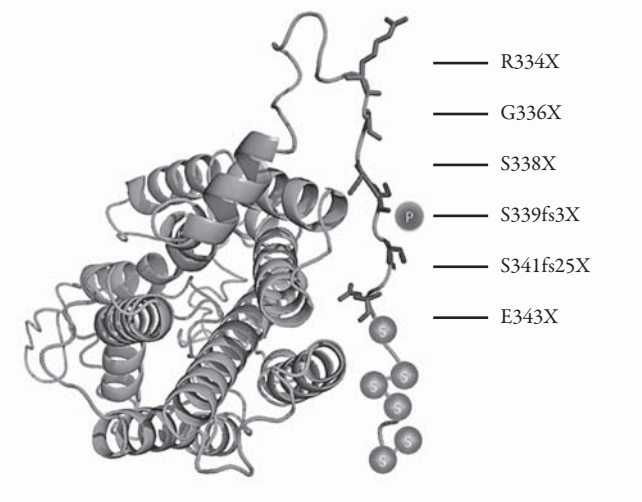

Figure 40.2 *Distribution of mutations in the cytoplasmic tail of CXCR4.* A model of CXCR4 is shown from the cytoplasmic face of the receptor parallel to the transmembrane helices in green. Helix 8, which runs parallel to the plasma membrane, is shown in orange. The positions of residues that are mutated to termination codons (red) or frameshifted to introduce missense peptides (blue) are indicated at right. A serine residue (S339) demonstrated to be phosphorylated by receptor activation (Woerner et al., 2005) is indicated by the purple sphere. Distal serines are indicated by green spheres. (See Color Plate.)

signaling (Bleul et al., 1998), signaling through phosphoinositide-3 kinase (PI-3K) isoforms (Ganju et al., 1998), and signaling through phospholipase C isoforms (Guinamard et al., 1999; Haribabu et al., 1997; Roes et al., 2003). CXCR4 dimerization following activation can also trigger the JAK2/JAK3/STAT signaling pathway (Vila-Coro et al., 1999). These upstream signaling mediators activate a cascade of downstream effectors, including ERK1/2 (Ganju et al., 1998) and Akt (Sotsios et al., 1999). Activation of these pathways ultimately drives the chemotactic response or results in transcriptional changes associated with differentiation or survival (summarized in Fig. 40.3) in a cell-specific fashion.

Following receptor activation, downregulation of CXCR4 signaling occurs via serine-threonine phosphorylation of the cytoplasmic tail domain by GPCR kinases and β-arrestin–mediated internalization characteristic of GPCR desensitization (Luttrell and Lefkowitz, 2002). The specific GPCR kinase involved in regulating CXCR4 was initially speculated to be GRK6 based on the neutrophil phenotype in null mice. Bone marrow neutrophils from these animals showed enhanced CXCL12-induced chemotaxis and impaired desensitization of the calcium response in vitro (Vroon et al., 2004). However, GRK3 appears to be more relevant in humans as it is specifically induced by a cytokine (GM-CSF) that downregulates CXCR4 (Wang et al., 2001) and has been shown to be transcriptionally deficient in WHIM syndrome (Balabanian et al., 2008).

The role of the CXCR4 cytoplasmic tail in receptor downregulation and internalization has been extensively characterized by the generation of a series of truncation mutants. Complete or partial truncation of the 47-residue tail domain enhances calcium signaling and abolishes receptor desensitization in response to CXCL12 (Amara et al., 1997; Haribabu et al., 1997). CXCR4 mutants lacking the cytoplasmic tail are refractory to β-arrestin–mediated regulation of G-protein signaling, although regulation can be recovered by the overexpression of β-arrestin 2, potentially via β-arrestin–receptor interactions at an internal receptor loop (Orsini et al., 1999). These defects in signaling desensitization were paralleled by complete or partial impairment of receptor internalization (Amara et al., 1997; Haribabu et al., 1997; Signoret et al., 1997). Mutants with more limited deletions of 23 or 12 residues had intermediate effects, suggesting that multiple residues along the tail domain contributed to internalization efficiency (Signoret et al., 1997). Serine-to-alanine point mutants in the tail domain also impaired ligand-mediated chemotaxis to variable degrees but did not completely abolish internalization (Orsini et al., 1999). The dileucine motif has been identified as an ubiquitinylation site, establishing trafficking to the proteasome as a mechanism for modulation of CXCR4 signaling (Fernandis et al., 2002; Marchese and Benovic, 2001).

CXCR4 internalization can also be regulated in a ligand-independent fashion. Heterologous desensitization occurs in response to phorbol esters (Haribabu et al., 1997; Signoret et al., 1997), cytokines (Nagase et al., 2002; Wang et al., 2001) (G/GM-CSF, IFN-α/γ, IL-4/13), and other chemokines such as CXCL8 (Richardson et al., 2003). Heterologous desensitization of CXCR4 can also occur in the absence of receptor internalization, as described for the cytokines IL-10 (Balabanian et al., 2002) and IL-16 (Van Drenth et al., 2000). This mechanism represents a potential mechanism for modulation of signaling inputs in a cytokine-rich environment such as the lymph node.

CXCR4 FUNCTION IN HEMATOPOIETIC LINEAGES

In mice lacking CXCR4 or CXCL12, fetal liver progenitor cells failed to home to the bone marrow (Ma et al., 1998; Nagasawa et al., 1996a; Zou et al., 1998), highlighting the critical importance of this receptor/ligand pair in driving localization of neutrophils to the appropriate developmental niche. This phenotype was also observed in irradiated mice reconstituted with donor cells derived from *Cxcr4*$^{-/-}$ fetal liver, with diminished numbers of bone marrow neutrophils and elevated numbers of immature neutrophils in the peripheral blood (Ma et al., 1999). In the postnatal period, CXCR4 continues to play a critical role in neutrophil mobilization from and back to the bone marrow (Levesque et al., 2003; Suratt et al., 2004). Ex vivo human peripheral neutrophils express low levels of cell-surface CXCR4, but the concentration of receptors on the cell surface increases after a short period of culture, a process that can be regulated by various cytokines (Nagase et al., 2002). Studies of murine neutrophils suggest that CXCR4 cell-surface expression is downregulated as neutrophils mature, thus suppressing CXCR4/CXCL12 signaling and permitting mobilization from the bone marrow, then upregulated in the periphery to facilitate the homing

Table 40.2 FREQUENCY OF DIFFERENT GENETIC ETIOLOGIES CAUSING WHIM SYNDROME

GENE	MUTATION	FAMILIAL	CASES	REFERENCE
CXCR4	R334X	+	6	Hernandez et al. (2003)
		+	4	Hernandez et al. (2003)
		+	3	Siedlar et al. (2008)
		+	2	Hernandez et al. (2003)
		+	2	Hernandez et al. (2003)
			1	Hagan & Nguyen (2007)
			1	Tarzi et al. (2005)
			1	Gulino et al. (2004)
			1	Tassone et al. (2009)
			1	Tassone et al. (2009)
			1	Tassone et al. (2009)
	G336X	+	2	Gulino et al. (2004)
	S338X	+	4	Vinurel et al. (2008)
		+	2	Balabanian et al. (2005)
		+	2	Tassone et al. (2009)
			1	Alapi et al. (2007)
	S339fs342X	+	2	Hernandez et al. (2003)
			1	Sanmun et al. (2006)
			1	Tassone et al. (2009)
	S341fs365X		1	Tassone et al. (2009)
	E343X	+	2	Hernandez et al. (2003)
			(n = 41)	
GRK3		+	2	Balabanian et al. (2008)
Unknown		+	2	Aprikayan et al. (2000)
			2	Aprikayan et al. (2000)
			1	Latger-Cannard et al. (2006)
			1	Balabanian et al. (2005)
			1	Imashaku et al. (2002)
			1	Arai et al. (2000)
			1	Goddard et al. (1994)
			(n = 9)	

of senescent neutrophils to the bone marrow (Martin et al., 2003).

CXCR4 also plays a critical role in B-cell development and trafficking. Mature B cells are absent from *Cxcr4*-null (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998) and *Cxcl12*-null (Nagasawa et al., 1996b) mouse embryos. CXCL12/CXCR4 signaling is critical for B-cell development from the earliest stages of differentiation along this lineage (Egawa et al., 2001). As in neutrophils, chemotaxis and calcium signaling undergo a differentiation-dependent downregulation, with response to ligand diminishing in maturing B cells despite continued robust cell-surface receptor expression (Fedyk et al., 1999; Honczarenko et al., 1999; Wehrli et al., 2001).

Following maturation in the bone marrow, B lymphocytes circulate between blood, lymphoid (lymph node, spleen, Peyer's patches), and extralymphoid compartments in a chemokine-directed fashion (Kim and Broxmeyer, 1999). Normal recirculation involves the reacquisition of CXCL12 responsiveness in order to allow the proper trafficking of B cells into and within lymphoid organs. Access to the lymphoid compartment involves transendothelial migration across specialized postcapillary high endothelial venules by a well-coordinated series of adhesive interactions involving L-selectin–mediated rolling and chemokine-mediated firm adhesion (Moser and Loetscher, 2001). CCR7 plays a primary role in this process, but CXCR4 serves an important, though redundant, role (Okada et al., 2002). Of note, L-selectin signaling in lymphocytes induces the cell-surface mobilization of internalized stores of CXCR4, potentiating CXCL12-mediated transmigration across high endothelial venules (Ding et al., 2003).

After migration into lymph node germinal centers and antigen-driven maturation, differentiated memory B cells and

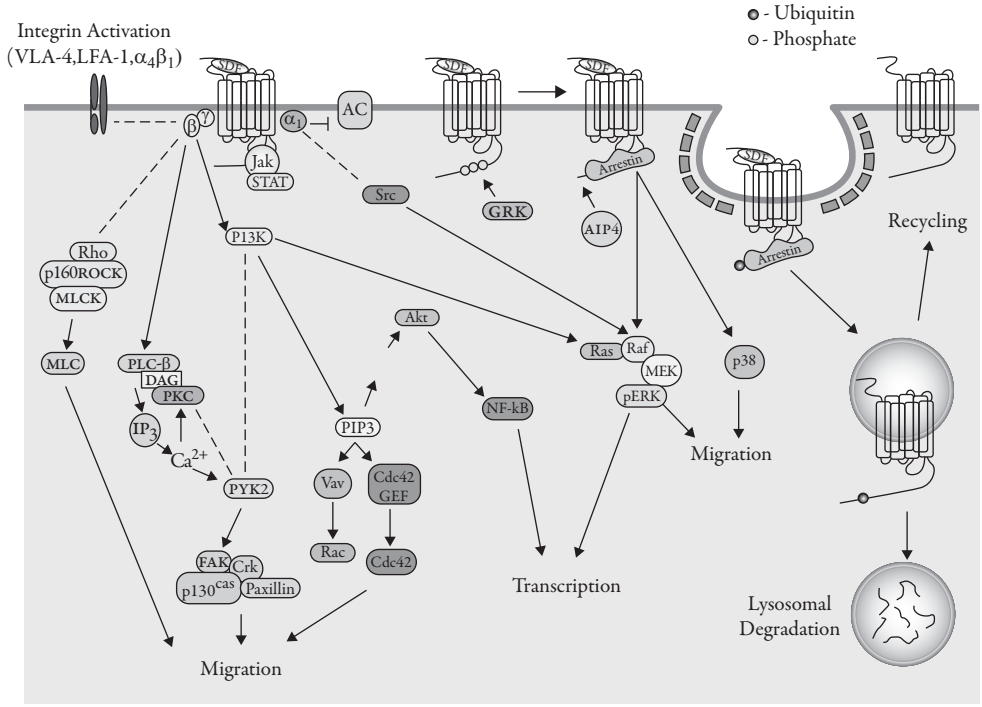

Figure 40.3 *Overview of CXCR4 signaling pathways.* Cartoon of the various signal transduction pathways that have been reported to be activated following receptor ligation with CXCL12. Downregulation by β-arrestin/CXCR4 interaction results in endocytosis of the receptor and intracellular trafficking resulting in recycling to the cell surface or degradation. (Adapted from a review by Busillo and Benovic, 2007.) (See Color Plate).

plasmablasts migrate to medullary cords, exit via the efferent lymph, and ultimately home to bone marrow, epithelia, or inflammatory sites. During the course of maturation, germinal-center B cells become unresponsive to CXCL12 (Bleul et al., 1998; Vicente-Manzanares et al., 1998). Loss of chemotactic responsiveness has been proposed to be important for the regulated migration of plasmablasts from lymph nodes (Wehrli et al., 2001). Mature, antibody-secreting plasma cells regain chemotactic responsiveness to CXCL12 (Hargreaves et al., 2001; Nakayama et al., 2003) and lodge in tissues (splenic red pulp, lymph node medullary cords, bone marrow) that express high levels of CXCL12.

DISEASE PATHOPHYSIOLOGY

Given the narrow spectrum of *CXCR4* mutations identified to date in WHIM syndrome, an attractive model to explain the disease pathophysiology focused on the role of the carboxyl terminal tail domain in receptor downregulation. As anticipated, based on studies of CXCR4 truncations characterized in vitro, disease-associated mutations enhanced intracellular release calcium, actin polymerization, and chemotaxis (Balabanian et al., 2005; Gulino et al., 2004; Hernandez et al., 2003). A receptor internalization defect has been demonstrated in both transfected cell systems and patient-derived cells, confirming the relevance of this mechanism to disease pathogenesis (Balabanian et al., 2005; Gulino et al., 2004; Tassone et al., 2009). This conclusion is supported by the observation that CXCR4 transduced into cultured dermal fibroblasts derived from WHIM syndrome patients lacking *CXCR4* mutations failed to internalize following stimulation, an observation subsequently explained by deficient *GRK3* expression (Balabanian et al., 2005, 2008). The mechanism of this internalization defect has been explored further and the enhanced signaling has been suggested to also involve prolonged β-arrestin 2-dependent signaling (Lagane et al., 2008).

Based on the current evidence, a plausible model explaining the pathophysiology of myelokathexis can be constructed. Recent models proposed to describe the regulation of neutrophil mobilization from the bone marrow stroma suggest that the reciprocally regulated activities of CXCR4 and CXCR2 are the major determinants in trafficking from and to the bone marrow (Martin et al., 2003; Suratt et al., 2004). The data on the effects of the truncating mutations found in WHIM syndrome neutrophils suggest that mutant cells respond inappropriately to stromal CXCL12 with respect to chemotactic response and potentially to adhesiveness (Gulino et al., 2004). The inhibitory effect of CXCR4 signaling on CXCR2 would render the mutant neutrophils unable to respond as robustly to CXCR2 ligands as necessary to drive trafficking out of the bone marrow compartment, effectively sequestering them. Inflammatory mediators such as cytokines or other chemokines that inhibit CXCR4 activity through heterologous desensitization would alleviate this inhibition of CXCR2, allowing egress of mature neutrophils (Fig. 40.4). It is also possible that the signaling abnormalities caused by the mutant receptor also have an effect on neutrophil apoptosis. Accelerated apoptosis compared to control samples has been observed in peripheral and bone marrow neutrophils (Aprikyan et al., 2000; Taniuchi et al., 1999), consistent with such a mechanism. Nonetheless,

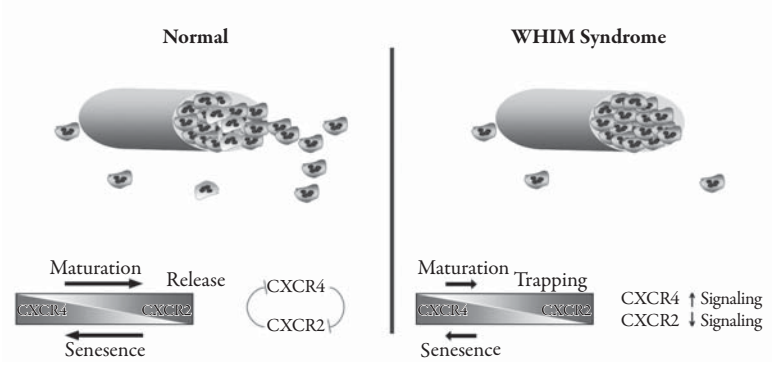

Figure 40.4 *Model for the mechanism leading to the myelokathexis phenotype in WHIM syndrome.* The downregulation of CXCR4 and upregulation of CXCR2 during normal neutrophil maturation is shown at left with the counterregulatory relationship of CXCR4 and CXCR2 diagrammed. In WHIM syndrome, the overactivity of the mutant CXCR4 receptor predominates in the CXCR2/CXCR4 axis, effectively keeping mutant neutrophils sequestered in the bone marrow unless a significant cytokine release, as in the setting of infection, causes heterologous desensitization of CXCR4 and permits CXCR2 signaling to drive chemotaxis to the periphery. (Adapted from Diaz and Gulino, 2005.) (See Color Plate).

the preponderance of evidence currently suggests that the trafficking defect is the principal mechanism explaining the myelokathexis of WHIM syndrome.

The disease pathophysiology for the immunological and HPV-associated aspects of the disorder is less clear. Lymphocytes are not sequestered in the bone marrow of WHIM patients, and while there have been no descriptions of morphological changes suggestive of apoptosis, it is not apparent whether the observed lymphopenia is due to trapping in an as-yet-uncharacterized compartment or due to premature apoptosis. The susceptibility of WHIM syndrome patients to HPV infection is also poorly characterized. Of note, *CXCL12* expression in HPV-infected dermis from WHIM syndrome patients and normal controls was upregulated, raising the possibility that enhanced CXCR4 signaling could represent a host susceptibility factor favorable for HPV persistence (Balabanian et al., 2005).

DIAGNOSIS AND TREATMENT

DIAGNOSIS

Diagnosis of WHIM syndrome in patients with the full spectrum of clinical features is relatively straightforward, but the variability observed in the disorder can lead to overlap with other conditions. Patients with a history of immunodeficiency and warts might be considered for the diagnosis if significant (<1,000/mL) and noncyclical neutropenia can be demonstrated. Features that suggest the presence of myelokathexis and the need for a bone marrow biopsy include reactive leukocytosis with infection and rapid response to granulocyte growth factors within hours. The absence of myelokathexis excludes the diagnosis (Fig. 40.5). A family history consistent with dominant inheritance is suggestive, as is a history of cardiac malformation. The diagnosis is made on clinical grounds if all features are present, but mutation analysis is useful to characterize disease as *CXCR4*wt or *CXCR4*mutant. If only a subset of clinical features is present, the likelihood of detecting a *CXCR4* mutation is significantly lower (Tassone et al., 2009). In patients with confirmed *CXCR4* mutations, neutropenia and lymphopenia, particularly B lymphopenia, appear to be universally present but HPV and hypogammaglobulinemia are quite variable over the lifetime of a patient. A diagnostic algorithm for evaluating potential WHIM syndrome cases is shown in Figure 40.5.

The list of potential phenocopy diseases with hematological and immune deficits similar to those observed in WHIM syndrome (neutropenia, B lymphopenia, variable T-cell dysfunction) is relatively short (Cham et al., 2002): common variable immunodeficiency (CVID), IgA deficiency, reticular dysgenesis, and class-switch recombination defect syndromes. The genetic etiologies have been described for class-switch recombination defects (*CD40* gene, encoding the CD40 protein and *CD40L* gene, encoding CD40 ligand), cartilage-hair hypoplasia (*RMRP*, a structural RNA), and Griscelli syndrome (*MYO5A*, encoding a myosin motor; *RAB27A*, encoding a vesicular transport protein). The spectrum of gene defects that can cause this constellation of hematological findings is diverse, and the pathophysiological basis of the shared hematological phenotype is not readily apparent.

TREATMENT AND PROGNOSIS

To date, treatment regimens described for WHIM syndrome patients have involved the use of neutrophil mobilizing agents (G-CSF or GM-CSF), intravenous immunoglobulin (IVIG), and prophylactic antibiotics individually or in combination. The efficacy of each of these approaches is not possible to assess currently with any confidence. There is a rational basis for employing G-CSF and/or GM-CSF, as the direct stimulation of neutrophil elastase release from myeloid precursors results in cleavage of both CXCR4 and CXCL12, facilitating myeloid-cell release from the bone marrow (Petit et al., 2002; Valenzuela-Fernandez et al., 2002). Both have been used to elevate neutrophil counts in confirmed WHIM syndrome cases (Alapi et al., 2007; Hord et al., 1997; Siedlar et al., 2008; Tassone et al., 2009; Wetzler et al., 1992), but efficacy is not

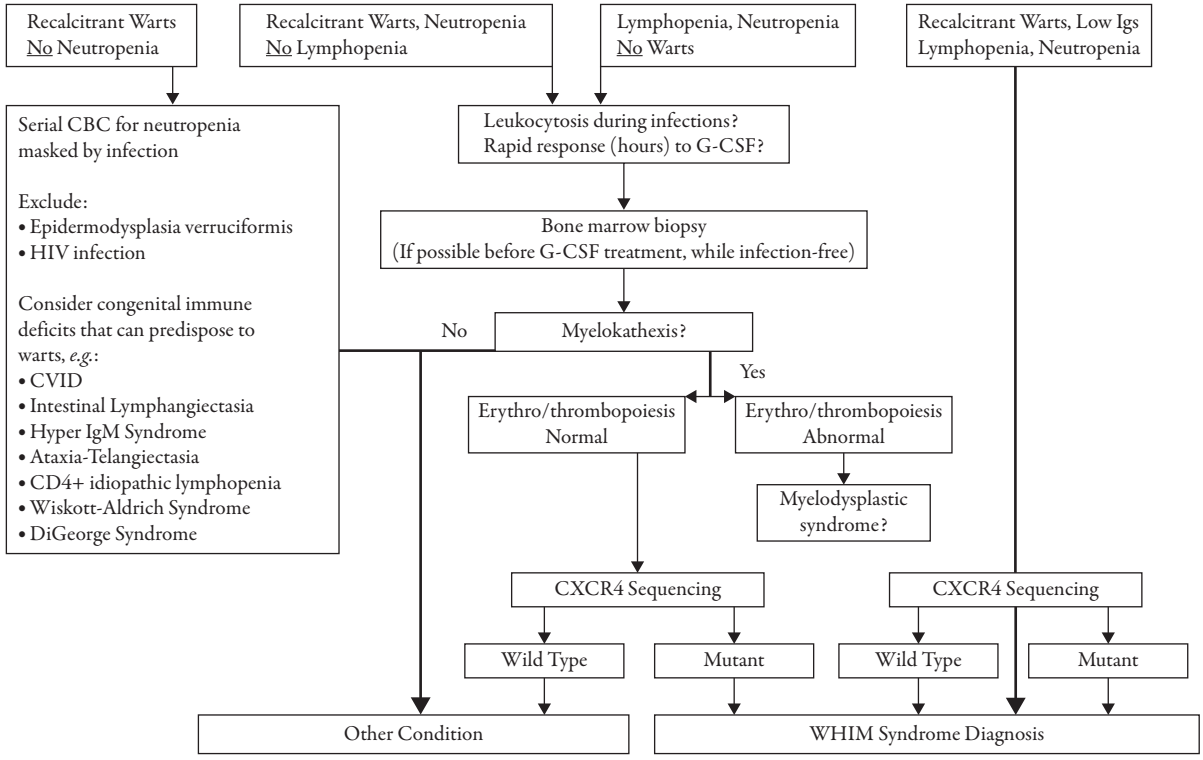

Figure 40.5 *Diagnostic algorithm for the evaluation of suspected WHIM syndrome cases.* A potential approach for considering the diagnosis of WHIM syndrome in patients with neutropenia and features suggestive of a combined immunodeficiency. G-CSF, granulocyte colony-stimulating factor. (Adapted from Diaz and Gulino, 2005.)

well established. Similarly, there are available data to suggest that WHIM patients who are having frequent bacterial infections will benefit from treatment with IVIG. Good control of recurrent bacterial infections has been reported with the institution of IVIG therapy (Goddard et al., 1994; Siedlar et al., 2008; Wetzler et al., 1990), but the rarity of the disease has precluded statistically rigorous approaches to testing efficacy. With an understanding of the molecular pathogenesis of the disease, the opportunity exists to capitalize on the development of CXCR4 inhibitor molecules developed for use in the treatment of HIV.

Regardless of which, if any, therapy was employed, the clinical outcomes for most of the patients described to date have been good. However, in light of the growing number of cases in which neoplasia has developed, surveillance for EBV-associated cancers is certainly warranted. Ongoing gynecological surveillance for potential malignant transformation of genital papillomavirus lesions is also essential.

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41.

PULMONARY ALVEOLAR PROTEINOSIS

Luigi D. Notarangelo

Pulmonary alveolar proteinosis (PAP) is characterized by accumulation of surfactant in alveolar macrophages, resulting in respiratory insufficiency. Patients with PAP also present with abnormalities of myeloid cells leading to increased susceptibility to infections.

The surfactant is composed of lipids and proteins (surfactant proteins [SP]) and is produced by type II alveolar epithelial cells (AEC-II). Lipids (mostly phospholipids) make up 90 percent of surfactant and form bilayers that reduce surface tension at the air–liquid interface in the alveoli, thus avoiding alveolar collapse. Surfactant also contains hydrophobic proteins (SP-B, SP-C) that contribute to surfactant organization (Weaver and Conkright, 2001), as well as hydrophilic proteins (SP-A, SP-D) with antimicrobial properties (Kingma and Whitsett, 2006). Careful balance of surfactant production, reuptake, recycling, and catabolism by AEC-II and by alveolar macrophages maintains the surfactant pool at relatively stable levels.

Conditions with surfactant metabolism dysfunction in humans include disorders of surfactant production (DSP) and PAP. In the former, the composition of surfactant is affected because of mutations in genes that encode for SP-B, SP-C (Whitsett and Weaver, 2002) or for the ATP-binding cassette subfamily A member 3 (ABCA3) protein, a membrane lipid transporter (Shulenin et al., 2004). As a result of altered composition of surfactant and inability to lower alveolar surface tension, alveolar anatomy is distorted in patients with DSP, leading to respiratory failure. In contrast, surfactant composition is normal and alveolar architecture is largely preserved in patients with PAP, but surfactant accumulates in alveolar macrophages and in alveolar walls, leading to inflammatory changes (Trapnell et al., 2009). A series of observations in humans and mice have shown that defects of granulocyte macrophage-colony stimulating factor (GM-CSF)–mediated signaling play a critical role in the pathophysiology of PAP, regardless of the congenital, autoimmune, or secondary nature of the disorder.

MOLECULAR PATHOPHYSIOLOGY OF PAP

The molecular pathophysiology of PAP was unraveled following the serendipitous discovery that disruption of the *Csf2* gene (encoding for GM-CSF) in mice leads to a lung pathology that is similar to human PAP, with accumulation of foamy alveolar macrophages, periodic acid-Schiff (PAS)–positive material in alveolar walls, and peribronchial lymphocytic infiltrate (Dranoff et al., 1994; Stanley et al., 1994). While the material that accumulates in alveolar macrophages and walls was shown to be surfactant, and its composition to be normal, the accumulation of surfactant was found to be due to reduced clearance by alveolar macrophages; reuptake, recycling, and catabolism by AEC-II are not affected (Dranoff et al., 1994). Similar features were subsequently demonstrated in mice in which the *csf2rb* gene, encoding for the β chain of the GM-CSF receptor (GM-CSFR), had been disrupted (Robb et al., 1995).

Following this discovery, the effect of GM-CSF on the function of alveolar macrophage has been studied extensively. GM-CSF binds to a heterodimeric cell-surface receptor (GM-CSFR) that includes a GM-CSF–binding α chain and a signal-transducing common β chain (β_c) shared by the receptors for interleukin (IL)-3 and IL-5 and known to bind the intracytoplasmic tyrosine kinase JAK2 (Hercus et al., 2009). Upon ligand binding, a dodecahedral complex is formed that consists of four molecules each of GM-CSFRα, β_c, and JAK2 molecules (Hansen et al., 2008). At low GM-CSF

concentrations, signaling through GM-CSFR results in myeloid cell survival and differentiation via activation of NF-κB and induction of bcl-2, whereas higher GM-CSF concentrations induce STAT5 phosphorylation and result in cellular activation and proliferation. This dual effect is mediated by β_c phosphorylation at residues Ser^{585} or Tyr^{577}, respectively (Guthridge et al., 2006). Importantly, the levels of GM-CSF in the lungs regulate expression of the transcription factor PU.1 in alveolar macrophages (Fig. 41.1) and promote adhesion, phagocytosis, expression of various surface receptors (Toll-like receptor [TLR]-2, TLR-4, Fc receptors, α- and β-integrins), secretion of inflammatory cytokines, microbial killing, and catabolism of surfactant (Shibata et al., 2001). These functions were defective in alveolar macrophages obtained from *csf2*$^{-/-}$ mice (Paine et al., 2001; Shibata et al., 2001) but could be rescued by retroviral-mediated expression of PU.1 (Shibata et al., 2001). Furthermore, the PAP pathology observed in *csf2*$^{-/-}$ mice can be rescued by restoring GM-CSF levels in the lungs, but not by its systemic administration (Huffman et al., 1996; Reed et al., 1999), indicating a critical role for pulmonary GM-CSF in alveolar macrophage function and surfactant homeostasis. Moreover, the lung phenotype of *csf2rb*$^{-/-}$ mice can be rescued by transplantation of bone marrow cells from wild-type donors (Nishinakamura et al., 1996) or of β_c-deficient bone marrow cells transduced with a β_c-expressing retroviral vector (Kleff et al., 2008), indicating the critical role of lung macrophages (of hematopoietic origin) rather than AEC-II cells in the pathophysiology of the disease.

GM-CSF–mediated signaling brings together innate and adaptive immune responses. It induces secretion of IL-18 and IL-12 by macrophages. These cytokines stimulate Th1 and NK lymphocytes to produce IFN-γ, thus prompting immune responses against intracellular pathogens (Berclaz et al., 2002). In keeping with this, *csf2*$^{-/-}$ mice are highly susceptible (and show increased mortality) to a variety of pathogens, including *Streptococcus*, *Pseudomonas*, *Listeria*, *Pneumocystis jiroveci*, *M. tuberculosis*, and adenovirus (Ballinger et al., 2006; Carey et al., 2007; Gonzalez-Juarrero et al., 2005; LeVine et al., 1999; Shibata et al., 2001). These infections are not strictly confined to the lungs, indicating that impaired GM-CSF–mediated signaling results in systemic defects of immune responses. Altogether, these data from *csf2*$^{-/-}$ mice clearly indicated that GM-CSF plays a critical role in the regulation of alveolar macrophage differentiation and function and in immune defense. Furthermore, the striking similarities in lung pathology between *csf2*$^{-/-}$ mice and patients with PAP suggested that congenital or acquired abnormalities of GM-CSF–mediated signaling play a role in the pathophysiology of the disease in humans.

PAP: CLASSIFICATION AND MOLECULAR FEATURES

The first case of PAP in humans was reported in 1958 (Rosen et al., 1958), but its pathogenesis remained obscure for several decades until *csf2*$^{-/-}$ mice were generated and characterized. Current classification of human PAP includes autoimmune, congenital, and secondary forms (Trapnell et al., 2009).

In 1999, Kitamura et al. reported neutralizing anti–GM-CSF autoantibodies in the serum of patients with PAP (Kitamura et al., 1999). Autoimmune PAP accounts for 90 percent of all cases of PAP (Inoue et al., 2008), yet its prevalence is low (3 or 4 individuals per million). Usually, clinical manifestations begin in the third or fourth decade of life, and lung

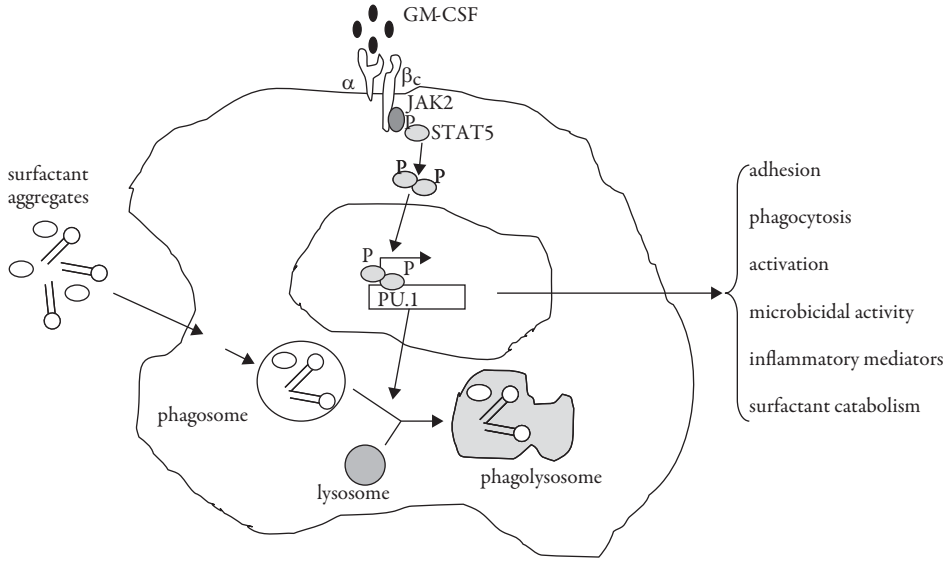

Figure 41.1 *GM-CSF–mediated activation of alveolar macrophages.* GM-CSF binds to its receptor, composed of α and β_c chains, expressed on the surface of alveolar macrophages. This promotes phosphorylation of JAK2 and recruitment and phosphorylation of STAT5. Phosphorylated STAT5 homodimers translocate to the nucleus and drive PU.1 gene transcription. Expression of the PU.1 transcription factor promotes phagocytosis and catabolism of surfactant aggregates within phagolysosomes, as well as cellular adhesion, production of microbicidal molecules, and release of inflammatory mediators.

pathology is characterized by accumulation of PAS-positive material in the alveoli that maintain a normal architecture. Patients with autoimmune PAP are also at high risk of infections, which account for 18 percent of the deaths (Seymour and Presneill, 2002). The antibodies detected in patients with autoimmune PAP bind GM-CSF with high affinity (Uchida et al., 2004). Interestingly, it has recently been shown that GM-CSF autoantibodies are present also in healthy donors (Uchida et al., 2009) and in pharmaceutical immunoglobulin preparations (Svenson et al., 1998). However, the levels of GM-CSF autoantibodies are much higher in patients with autoimmune PAP, leading to the hypothesis that they may play a pathogenic role when their concentration exceeds a certain threshold (Bendtzen et al., 2007), estimated to be more than 10.4 μg/m: (Uchida et al., 2009). Injection of GM-CSF autoantibodies isolated from patients with autoimmune PAP into nonhuman primates can in fact reproduce the features of the disease if autoantibody levels are maintained above 40 μg/mL for several months (Sakagami et al., 2009).

Following the demonstration that disruption of the *csf2* and *csf2rb* genes in mice leads to a PAP phenotype, defects in the same genes were sought in humans with PAP. In 1997, four infants with an established or putative diagnosis of PAP were reported in whom lack of β_c expression was demonstrated on the surface of peripheral leukocytes (Dirksen et al., 1997). Expression of the GM-CSFRα chain was preserved, but binding of GM-CSF and in vitro progenitor clonogenic assays showed altered response to GM-CSF. Homozygosity for a single amino acid substitution (Pro603Thr) was identified in one of the three patients studied, but this was then found to represent a polymorphism (Freeburn et al., 1998). No defects in the *CSF2RB* gene were identified in the remaining patients, leaving the molecular basis of the disease unclear. However, more recently, a homozygous missense mutation in the *CSF2RB* gene, resulting in S271L amino acid substitution, was identified in a female child presenting with pneumonia and progressive dyspnea. The mutation prevented STAT5 phosphorylation in blood leukocytes following stimulation by GM-CSF and IL-3 (Suzuki et al., 2011). While this observation extends to humans previous observations in mice that *csf2rb* mutations may lead to a PAP phenotype, this remains a very rare cause of the disease.

In contrast, two groups have provided conclusive evidence that PAP may be caused by genetic defects of the *CSF2RA* gene, which is located in the pseudoautosomal region of chromosome X, at Xp22.3, and at the tip of the short arm of chromosome Y, at Yp11.32. Susuki et al. (2008) described two sisters aged 6 and 8 years with growth failure, tachypnea, and severe pulmonary restrictive impairment. Chest radiograph and histopathological examination of a lung biopsy were consistent with the diagnosis of PAP. Search for GM-CSF autoantibodies was negative. The concentrations of GM-CSF in the bronchoalveolar lavage (BAL) fluid, and of SP-D in the serum, were markedly elevated. Although expression of both GM-CSFRα and β_c chains at the surface of leukocytes was normal, GM-CSF–induced upregulation of CD11b was impaired and expression of a low-molecular-weight form of GM-CSFRα chain was demonstrated by Western blotting. Genetic analysis revealed that both sisters carried a 1.6 Mb deletion in the pseudoautosomal region 1 of the maternally derived X chromosome and a single nucleotide mutation, leading to Gly174Arg substitution, in the paternally derived *CSF2RA* gene. When co-transfected with β_c into 293 cells, the GM-CSFRα chain mutant failed to internalize GM-CSF added to the culture and resulted in markedly decreased phosphorylation of STAT5 in response to GM-CSF. This defect was partially rescued at higher GM-CSF concentrations (Suzuki et al., 2008). Martinez-Moczygemba et al. described a 4-year-old girl with Turner syndrome and respiratory insufficiency; pathological findings at lung biopsy and on BAL were also consistent with the diagnosis of PAP. Search for GM-CSF autoantibodies was negative, but serum GM-CSF concentration was increased more than 200-fold. A complete lack of GM-CSFRα chain expression on the surface of peripheral blood monocytes was demonstrated, and expression of CD11b was not upregulated in response to GM-CSF. Molecular cytogenetic analysis showed a 46Xi(Xq) karyotype, with truncation of the Xp arm and lack of pseudoautosomal region 1 on the i(Xq) chromosome. The observation that the GM-CSFRα chain transcript could not be demonstrated by RT-PCR prompted a search for mutation on the other allele of the *CSF2RA* gene; a deletion of the genomic segment encompassing exons 5–13 was demonstrated (Martinez-Moczygemba et al., 2008). Altogether, these observations provide the first evidence that biallelic mutations of the *CSF2RA* gene cause congenital PAP in humans. Since then, seven additional children, all girls, have been identified with congenital PAP due to *CSF2RA* mutations (Trapnell et al., 2009). Elevated GM-CSF levels were consistently demonstrated in every patient.

Finally, PAP may also occur in association with various hematological disorders (myelodysplastic syndromes, leukemia, lymphoma, etc.), immunological diseases (severe combined immunodeficiency, IgA deficiency), infections (cytomegalovirus, *M. tuberculosis*, *P. jiroveci*, others), lysinuric protein intolerance, hematopoietic cell transplantation, and exposure to toxic substances and elements (Chung et al., 2009; Seymour and Presneill, 2002; Trapnell, 2003). The pathogenesis of secondary PAP remains poorly defined and may reflect acquired depletion or functional defects of alveolar macrophages.

CLINICAL FEATURES, DIAGNOSIS, AND TREATMENT

The main clinical features of PAP include progressive respiratory failure with hypoxemia and growth arrest. Chest radiographs and computed tomography show diffuse and patchy opacities (Ishii et al., 2009; Trapnell et al., 2003). Open lung biopsies demonstrate accumulation of granular PAS-positive proteinaceous material in the alveoli and in the alveolar walls, foamy macrophages, and inflammatory infiltrates, without disruption of the lung architecture. Foamy macrophages and PAS-positive material are also recovered following BAL. In

addition, patients with PAP suffer from increased susceptibility to pulmonary and extrapulmonary infections. These include both community- and hospital-acquired microorganisms (*S. pneumoniae*, *H. influenzae*, *Klebsiella*, *Pseudomonas*) and opportunistic pathogens (*Nocardia*, *Mycobacteria*). In a review of cases reported in the literature between 1958 and 1997, infections accounted for 18 percent of deaths (Seymour and Presneill, 2002).

Some clinical and laboratory features distinguish congenital PAP from autoimmune and secondary forms. The seven patients with molecularly proven *CSF2RA* mutations were 2 to 11 years old at the time of diagnosis (Trapnell et al., 2009); in contrast, autoimmune PAP typically affects subjects aged 30 to 40 years. Furthermore, no underlying disorders can be identified in patients with congenital PAP. At variance with autoimmune PAP, patients with congenital PAP do not have GM-CSF autoantibodies, and their GM-CSF serum levels are significantly elevated. In addition, levels of SP-D in the serum and of inflammatory chemokines and cytokines (MCP-1, GM-CSF, M-CSF) in BAL fluid are increased in patients with PAP, regardless of the congenital, autoimmune, or secondary nature of the disease (Trapnell et al., 2009).

Whole-lung lavage (i.e., large-volume BAL) is often used as the first line of treatment in patients with PAP and results in significant clinical and radiographic improvement (Michaud et al., 2009). However, approximately 15 percent of the patients (including those with congenital PAP) have a relapsing course and may require multiple rounds of the procedure. The observation that some patients with congenital PAP have residual GM-CSF–mediated signaling suggests that aerosolized delivery of GM-CSF might be therapeutic in these patients (Suzuki et al., 2008). Finally, because of the critical role of alveolar macrophage dysfunction in the pathogenesis of congenital PAP, hematopoietic stem cell transplantation (HSCT) and *CSF2RA* gene transfer might be considered. Indeed, one of the patients with congenital PAP due to *CSF2RA* mutations received HSCT from a matched unrelated donor but died from a severe respiratory infection shortly after transplantation, before immune reconstitution was achieved (Martinez-Moczygemba et al., 2008).

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42.

ROLE OF *TMC6* AND *TMC8* GENES AND EVER PROTEINS IN EPIDERMODYSPLASIA VERRUCIFORMIS

Maciej Lazarczyk, Patricia Cassonnet, and Michel Favre

Epidermodysplasia verruciformis (EV; OMIM #226400) is a very rare genodermatosis, initially described by Lewandowsky and Lutz in 1922. The nosological identity of the disease as well as its pathogenesis has long remained unclear (for reviews see Orth, 1987 and 2006). Some authors recognized EV as an abnormality of epidermal differentiation (Mashkilleisson, 1931; Oehlschlaegel et al., 1966; Waisman and Montgomery, 1942), whereas others treated it as a generalized form of skin warts (Hoffmann, 1926; Jablonska and Milewski, 1957; Kogoj, 1926; Sullivan and Ellis, 1939). A viral etiology of EV was proposed for the first time by Lutz in 1946 (Lutz, 1946). However, it was not until the 1960s that the presence of the virus in the skin lesions of EV patients was confirmed by electron microscopy, and the virus has subsequently been identified as a member of a specific group of widespread human papillomaviruses (HPV) (Jablonska et al., 1968; Orth et al., 1979; Ruiter and van Mullem, 1966). Malignant conversion of some verrucous lesions is frequently observed. This led to EV being considered as a model for the study of the role of papillomaviruses in oncogenesis (Jablonska et al., 1972). The first evidence for HPV etiology in human carcinogenesis was obtained in 1980 (Orth et al., 1980). All these findings suggested that EV might constitute in fact a defect of the natural mechanisms protecting humans from this specific group of oncogenic papillomaviruses.

Recently EV has been classified into the group of primary immune deficiencies, as a disease in which the defect would concern keratinocytes (Notarangelo et al., 2004). In this context, EV might be considered as the first described primary immunodeficiency (Orth, 2006), although the range of abnormalities observed in EV patients is broader, clearly not limited to the innate immunity.

CLINICAL COURSE OF EV

The first skin lesions emerge in infancy and the disease is usually diagnosed between the ages of 5 and 10 years. Initially, the lesions appear typically on the face and hands, but they subsequently spread to different regions, including the trunk. The lesions affect only the skin; the mucosal membranes are spared. Macroscopically the lesions in EV are classified as flat warts or macules. These lesions might resemble the ones found in the course of pityriasis versicolor, but they are persistent and can become confluent (Majewski et al., 1997; Orth, 2006). Some of the lesions, especially the ones in the sun-exposed areas, undergo malignant transformation, indicating that UV irradiation plays an important role in carcinoma. However, such a natural evolution of the lesion is slow, and nonmelanoma skin cancers (NMSC) emerge in EV patients typically after more than 20 to 30 years. Initially it has been estimated that only 30 percent of EV patients eventually develop NMSC (Lutzner, 1978), but more recent surveys, in which the EV patients have been observed for a long enough period (>40 years), demonstrate that skin cancers can emerge in virtually all of them (Majewski et al., 1997). Among the NMSC, squamous cell carcinoma (SCC) predominates. In EV, even though malignant, SCC only rarely produces remote metastases, unless the patients are exposed to irradiation (e.g., X-ray, UV light).

SUSCEPTIBILITY TO HPV

The constant and the most important characteristic feature of the phenotype associated with EV is an unusual susceptibility to infection by a specific group of evolutionary-related cutaneous HPV. Viruses that are responsible for the induction of

the skin lesions in EV patients belong to the beta genus (also called EV-HPV), and this group comprises more than 40 genotypes (Chouhy D et al., 2012). Initially it was thought that beta-HPV infections were restricted solely to EV patients. However, it has been subsequently demonstrated that these viruses are widespread, although the genetic material of the virus is detected in the general population only by highly sensitive techniques like nested PCR (Boxman et al., 1999). It implies that a natural anti-HPV barrier exists in humans and normally can eliminate or at least control beta-HPV infections. Conversely, the viral load in skin lesions in EV patients is high, suggesting that the molecular defect in EV apparently disrupts the barrier and consequently somehow promotes the life cycle of beta-HPV and/or prevents viral clearance (Lazarczyk et al., 2009).

Intriguingly, the high susceptibility to HPV in EV is extremely selective. EV patients are not more prone to other viral infections, including even those with closely related alpha-HPV—the group of papillomaviruses that comprises the genotypes causing cervical carcinomas (HPV16, HPV18, etc.). The reason for this puzzling selectivity, as well as the cause of the host restriction of beta-HPV, has long remained obscure, but recent reports have shed some light on this issue (Lazarczyk et al., 2008, 2009).

GENETIC BACKGROUND OF EV

The existence of familial aggregation of EV cases, especially in consanguineous families, very early drew the attention of the scientific community, and already in the 1930s the hereditary nature of EV was being considered. However, it was not until the 1970s that EV cases were more systematically investigated and characterized. The studies by Rajagopalan (Rajagopalan et al., 1972), followed by the observations of Lutzner (Lutzner, 1978), revealed an autosomal recessive type of EV inheritance, and currently this mode of genetic transmission of familial EV is considered as predominant. However, the EV locus and the incriminated genes were not discovered until 30 years later.

Two EV loci have been determined so far, on chromosomes 17 (EV1) and 2 (EV2), demonstrating a nonallelic heterogeneity of the disease (Ramoz et al., 1999, 2000). Subsequently, two adjacent genes in the EV1 locus have been identified (Ramoz et al., 2002). The genes belong to the *TMC* family (transmembrane channel-like), and the gene names are *TMC6* (*EVER1*) or *TMC8* (*EVER2*) (Keresztes et al., 2003). The *TMC* genes have been evolutionary conserved, and numerous *TMC* orthologs have been identified, not only in mammals but also in nonmammalian vertebrates (Keresztes et al., 2003). Mutations identified in EV so far are point substitutions, nucleotide insertions, or deletions (frameshift mutations) that create a premature STOP codon or deletion of exons (Table 42.1). All the EVER mutations are believed to prevent synthesis of full-length, functional protein capable of interacting with cellular partners such as the zinc transporter ZnT-1 (Lazarczyk et al., 2008). It is estimated that a nonsense mutation in *TMC* genes is found in around 70 to 80 percent of patients (Orth, 2006, 2008). The remaining, unequivocally diagnosed EV cases might carry a mutation in an as-yet-unidentified gene in the EV2 locus or in another chromosomal region. No mutation was detected in zinc transporter genes located in the EV2 locus (ZnT-6) or mapped to chromosome 1q32.3 (ZnT-1) (M. Favre, unpublished).

FUNCTION OF EVER PROTEINS

EVER genes are ubiquitously expressed, and they encode two related transmembrane proteins. EVER1 (805 amino acids [a.a.]) and EVER2 (726 a.a.) share ~28 percent of a.a.

Table 42.1 MAPPING OF EVER1 AND EVER2 MUTATIONS IN DIFFERENT EV PATIENTS

PATIENT	ORIGIN	GENE	MUTATION		REFERENCE
			NUCLEOTIDE	AMINO ACID	
1	Japan	EVER1	220C>T	Q74X	Aochi et al., 2007
2	Algeria	EVER1	280C>T	R94X	Ramoz et al., 2002
3	Japan	EVER1	744C>A	Y248X	Tate et al., 2004
4	Japan	EVER1	IVS8-2A>T	G298fsX1	Tate et al., 2004
5	China	EVER1	Ins916CATGT	Y306fsX12	Zuo et al., 2006
6	Pakistan	EVER1	del968T	L323fsX26	Gober et al., 2007
7	Colombia	EVER1	1726G>T	E576X	Ramoz et al., 2002
8	Algeria	EVER1	del1882A-2277T	delT630-N759	Favre and Kim, unpublished
9	Brazil	EVER2	188C>A	W63X	Rady et al., 2007
10	Poland	EVER2	IVS4-1G>T	T150fsX3	Lazarczyk et al., 2008
11	China	EVER2	568C>T	R190X	Sun et al., 2005
12	United States	EVER2	del561T-583A	delA188fsX71	Berthelot et al., 2007
13	Algeria	EVER2	del754T	F252fsX31	Ramoz et al., 2002
14	Colombia	EVER2	1084G>T	E362X	Ramoz et al., 2002

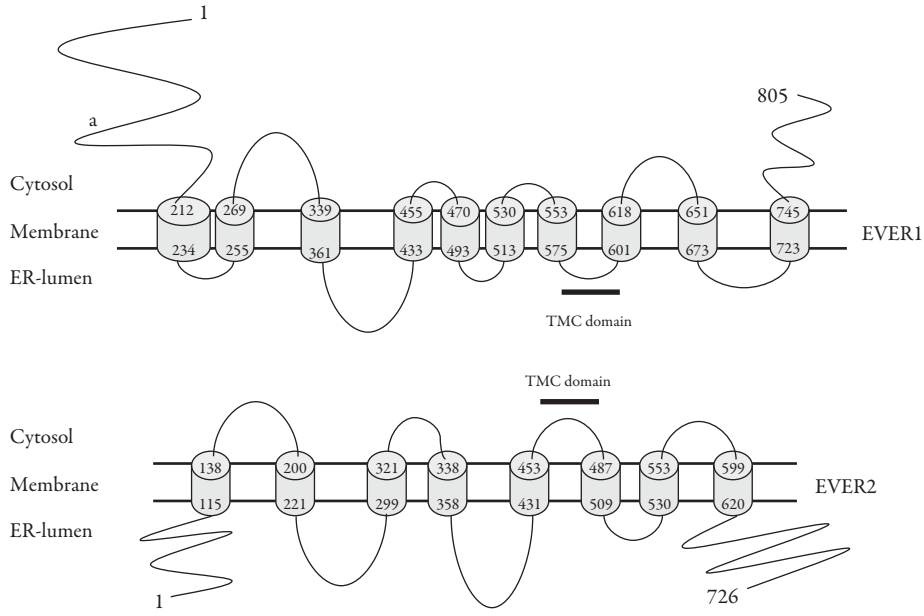

Figure 42.1 Schematic representation of EVER1 and EVER2 proteins. The putative cytosolic and lumenal regions and the conserved TMC domain of EVER1 (805 a.a.) and EVER2 (726 a.a.) are indicated. A.a. positions of the putative 10 (EVER1) and 8 (EVER2) transmembrane domains are shown.

(Ramoz et al., 2002) and are characterized by 10 and 8 putative transmembrane domains, respectively (Fig. 42.1). Both proteins have putative phosphorylation and glycosylation sites. The central part of the EVER proteins constitute the so-called TMC domain, the motif conserved in all the TMC proteins. In human keratinocytes, EVERs are located mainly in endoplasmic reticulum (ER), but also in the Golgi apparatus and nuclear membrane (Lazarczyk et al., 2008; Ramoz et al., 2002). Both EVER1 and EVER2 have been found to interact with zinc transporter 1 (ZnT-1), and the TMC domain of EVERs is necessary and sufficient for this interaction (Lazarczyk et al., 2008, 2009). The EVER complex probably comprises more proteins, as predicted by the yeast two-hybrid screening and bioinformatics analysis (Lazarczyk et al., 2009), but until now only the interaction with ZnT-1 has been unequivocally confirmed.

ZnT-1 was originally described as a plasma membrane zinc effluxer in hamster cells (Palmiter and Findley, 1995). However, in human keratinocytes, ZnT-1 was shown to form a complex with EVER1 and EVER2, which is located inside the cell (Lazarczyk et al., 2008; Lazarczyk and Favre, 2008). This complex does not confer zinc efflux in human keratinocytes but is probably involved in maintaining the low level of free zinc ions and in preventing zinc retention in the nucleus (Lazarczyk et al., 2008). The EVER/ZnT-1 complex is also involved in the regulation of activity of some cellular transcription factors, such as MTF-1 and AP-1. The exact role of the EVER/ZnT-1 complex in the transport or regulation of Zn^{2+} remains unknown, but undoubtedly, EVER1 and EVER2 constitute an element crucial for the complex integrity, as the lack either EVER compromises its activity (Lazarczyk et al., 2008). Recently, EVER2 protein was found to interact with TRADD to promote TNF-α induced apoptosis (Gaud G et al., 2013).

PATHOGENESIS OF EV

Despite the discovery of the EV susceptibility genes (Ramoz et al., 2002), the pathogenesis of the disease remains obscure. Even the cell type primarily affected by the EVER deficiency is uncertain; several possibilities can be considered. First of all, the lack of one of the EVER proteins in keratinocytes, the host cell for HPV, might directly promote the virus life cycle, allowing the virus to establish a persistent infection. On the other hand, EV patients display some immune deviations, in particular in cell-mediated immunity, and it is possible that EVER deficiency in lymphocytes affects their function and consequently the clearance of the HPV infection.

EVER PROTEINS AND THE HPV LIFE CYCLE

Most probably, the EVER/ZnT-1 complex is involved in keratinocytes in regulation of the cellular homeostasis of zinc (Lazarczyk et al., 2008, 2009; Lazarczyk and Favre, 2008). The total zinc in any cell can be classified in one of the two categories: (1) zinc tightly bound to different cellular macromolecules, in particular to metallothioneins, and (2) free zinc—the zinc ions that remain unbound or only loosely bound and are easily accessible (Eide, 2006; Lazarczyk and Favre, 2008). Even though the pool of free zinc remains in the low nanomolar range, the free zinc is metabolically very active and needed for the newly synthesized proteins. It can be assumed that the EVER/ZnT-1 complex is involved in maintaining a low concentration of free Zn^{2+} in the cell (Lazarczyk et al., 2008). Through limiting the accessibility of free zinc ions, the EVER complex could downregulate expression and replication of the virus in at least three different ways. First, many

viruses, including papillomaviruses, comprise zinc-binding proteins that require Zn^{2+} for their activity. In case of HPV, the E6 and E7 oncoproteins comprise two zinc fingers and one zinc finger, respectively (Barbosa et al., 1989). Second, Zn^{2+} affects cell signaling, or it can even serve as a classical second messenger (Yamasaki et al., 2007), and thus zinc ions may stimulate signaling pathways, leading to activation of some cellular transcription factors, including the ones relevant for the virus. Furthermore, by affecting the activity of several cellular transcription factors, zinc can change also the expression of multiple cellular genes and in this way indirectly influence the virus life cycle (for instance, by modifying the differentiation status of the cell, affecting proliferation, etc.). Currently it is unknown which of these tentative and not mutually exclusive mechanisms could explain the anti-HPV potential of the EVER complex. Verification of the role of the intracellular accessibility of free zinc ions for the viral proteins is technically challenging, but the scarcity of free Zn^{2+} in the cell (probably less than 1,000 atoms per cell), especially in the context of a massive synthesis of zinc-binding viral protein, prompts serious consideration of this mechanism. On the other hand, our data might support the last two explanations—that is, the effect of zinc via the influence on the cellular transcription factors and in turn on the cellular and viral gene expression pattern.

Indeed, EVER proteins serve as negative regulators of the activity of the AP-1 transcription factor family in human keratinocytes (Lazarczyk et al., 2008). Zn^{2+} can stimulate AP-1 by activation of Jun N-terminal Kinase (JNK) (Eom et al., 2001), an enzyme that phosphorylates and activates c-Jun and c-Fos. EVER proteins inhibit the transactivation domain of Jun, probably in a JNK-dependent but GSK-3beta-independent manner (Lazarczyk et al., 2008, and unpublished data). It has been demonstrated that c-Jun and c-Fos are essential for the HPV life cycle (Kyo et al., 1997). Therefore, in the EVER-deficient keratinocytes, where AP-1 activity is kept constitutively high (Lazarczyk et al., 2008), the HPV life cycle can be directly facilitated. However, since AP-1 is a transcription factor with pleiotropic activity, it can also affect the expression of multiple cellular genes, including the genes relevant for the virus itself, or those important for the clearance of the infection (for instance, cytokine expression). Indeed, EVER proteins were shown to regulate the expression of some interleukins produced by keratinocytes. Thus, the altered pattern of the cytokine production could contribute to the deregulation of the antiviral immune response and might enable persistent infection.

EVER PROTEINS IN LYMPHOCYTES

The efficient immune response against HPV plays an essential role in the natural history of the papillomavirus infections (clearance vs. persistence of lesions). Notably, even though the majority of women are infected at least once in their life with potentially oncogenic HPV, only in a small proportion of them does this infection lead to clinically apparent disease. Most frequently, the subclinical infections are spontaneously cured in immunocompetent individuals. It is believed that effective antigen presentation corresponding to E6 and E7 proteins and activation of T-cytotoxic lymphocytes are essential for such viral clearance (Tindle, 2002). It has been proposed that EV might constitute a disease in which the innate or adaptive immune response directed toward papillomaviruses is compromised. This might prevent elimination of the beta-HPV and lead to persistent infections. Consequently, a long-lasting exposure to the oncogenic viruses would provoke skin-cancer development in these patients.

Interestingly, the results of genome-wide transcriptome analysis (Su et al., 2004) revealed that the highest *EVER1* and *EVER2* expression takes place not in the skin but in lymphocytes, in particular in T cells (Lazarczyk et al., 2012). Therefore, it is tempting to speculate that the "defect" resulting from EVER deficiency concerns also the immune system. Namely, it has been postulated that the zinc imbalance, primarily reported in EVER-deficient keratinocytes (Lazarczyk et al., 2008), could be imposed by the lack of EVER also in lymphoid cells. Indeed, using a luciferase assay, it has been shown that activity of zinc-inducible transcription factor (MTF-1) is increased in lymphoblastoid lines with a mutation in EVER (Lazarczyk et al., 2009). Since an important role of zinc in maintaining the integrity of the immune system has repeatedly been raised during recent decades (Fraker and King, 2004; Rink and Gabriel, 2001), one can presume that the deregulation of zinc homeostasis might affect the lymphocyte function (Lazarczyk et al., 2012).

In line with these presumptions, multiple immune deviations have been reported in EV patients, although they remain poorly characterized. The response of the T lymphocytes from EV to unspecific mitogens and to HPV-infected keratinocytes is impaired (Cooper et al., 1990; Glinski et al., 1976), whereas the function of the B-cell compartment seems to be preserved (Prawer et al., 1977). Moreover, in the majority of EV patients, an anergy to contact sensitizers (mainly dinitrochlorobenzene) is observed (Glinski et al., 1976), although it is unclear which cellular compartment (dendritic cells, lymphocytes, etc.) contributes to this phenomenon. A distinct issue that remains is whether these immune abnormalities truly contribute to EV pathogenesis or whether they constitute a consequence of a massive, life-long HPV infection in EV patients.

It has been reported that some patients with severe combined immunodeficiency (SCID) develop EV-like lesions a few years after hemopoietic stem-cell (HSC) transplantation. HSC transplantation is a life-saving treatment in SCID patients, but, notably, it remains ineffective toward HPV-induced skin lesions (Laffort et al., 2004). This might disfavor the involvement of the dysfunction of the lymphocyte compartment in EV's pathogenesis, and it emphasizes the role of keratinocytes or NK cells. However, our recent results suggest that the EVER deficiency can have a direct influence on the immune cell function (Lazarczyk et al., 2012). Using the lymphoblastoid lines derived from EV patients or their healthy relatives, we have demonstrated in vitro that the lack of EVER2 correlates with increased MTF-1 transcription factor activity (Fig. 42.2) and TNF-alpha production (Lazarczyk et al., 2009). In accordance with these findings, increased expression of TNF-alpha in EV epidermis had previously been

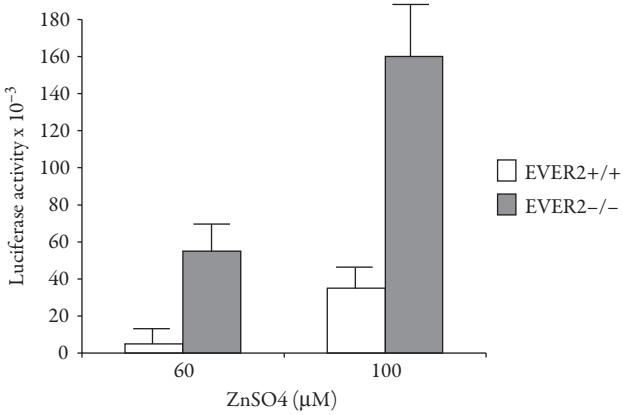

Figure 42.2 Activity of MTF-1 transcription factor is upregulated in mutant EVER2 lymphocytes. Lymphoblastoid cells with wild-type (+/+) or mutant (-/-) *TMC2* gene were transfected with the luciferase reporter gene under the control of the transcription factor MTF-1 in two different $ZnSO_4$ concentrations, as described in Lazarczyk et al., 2008.

reported (Majewski et al., 1991), although the significance of this cytokine in the pathogenesis of EV remains speculative.

SELECTIVITY OF EVER-BASED BARRIER

The reason of the remarkable selectivity of the susceptibility to beta-HPV in EV patients has long been unknown. This selectivity might suggest that the natural EVER-based barrier is highly selective and confers resistance solely to beta-HPV. However, our recent results support an alternative, virus-intrinsic mechanism. One of the important differences between beta-HPV and alpha-HPV is the lack of the E5 open reading frame (ORF) in the beta genotypes. E5 is a small hydrophobic protein with rather moderate transformation potential (Tsai and Chen, 2003), although it does contribute to HPV-mediated carcinogenesis (Maufort et al., 2007). It has been demonstrated that E5 binds to EVER proteins and disrupts the complex (Lazarczyk et al., 2008, 2009). Consequently, E5 exerts an effect strikingly similar to that of the EVER deficiency. Therefore, one can presume that the EVER-based barrier is not truly selective but conversely that some HPVs, different from beta-HPV, have developed a counteracting mechanism that allows them to break or bypass the natural anti-HPV barrier.

A MODEL OF A NATURAL ANTI-HPV BARRIER

Comprehension of EV pathogenesis, even though far from being complete, sheds light on the nature of the host–HPV interactions and gives an insight into the function of the natural mechanisms protecting humans from papillomaviruses. It allowed us to propose a model of the natural anti-HPV barrier (Lazarczyk et al., 2009). According to this model, an essential part of the barrier would be EVER proteins. The exact protein composition of the barrier remains to be established, but the substantial phenotype in EV patients clearly indicates that the presence of the EVER proteins is critical for the integrity of the barrier (Lazarczyk et al., 2008; Ramoz et al., 2002). From a cellular perspective, the anti-HPV barrier would comprise keratinocytes but possibly also the lymphocyte compartment (Lazarczyk et al., 2012). The barrier would be involved in maintaining the low level of free zinc and the low level of AP-1 activity.

In conclusion, deregulation of cellular zinc homeostasis, namely an increase in intracellular free zinc concentration, would constitute an important step in the virus life cycle not solely for EV-HPV but for HPV in general (Lazarczyk et al., 2009), and perhaps also for other viruses (Lazarczyk and Favre, 2008). However, two "strategies" would be employed: E5-mediated inhibition of the EVER complex in the case of alpha-HPV, or EVER/ZnT-1 complex function could be intrinsically compromised as an effect of the lack of one of the EVER proteins. Beta-HPVs, being devoid of E5, are obviously confined to the latter possibility, what imposes a host restriction for this group of papillomaviruses.

ACKNOWLEDGMENTS

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43.

WISKOTT-ALDRICH SYNDROME

Hans D. Ochs and Luigi D. Notarangelo

The first description of the Wiskott-Aldrich syndrome (WAS), published in 1937, defined accurately the clinical phenotype: three brothers, but not their sisters, presented shortly after birth with thrombocytopenia, bloody diarrhea, eczema, and recurrent ear infections (Wiskott, 1937). On the basis of this symptomatology, the early onset, and the predominance of male infants, Wiskott differentiated this entity from Morbus Werlhofii, a synonym for idiopathic thrombocytopenia (ITP). Seventeen years later, Aldrich described a large family with multiple affected males, clearly demonstrating X-linked inheritance (Aldrich et al., 1954). In 1957, additional cases were described (Huntley and Dees, 1957), including members of an African American family (Wolff, 1957).

Following these early clinical reports, the immunological abnormalities characteristic of WAS were recognized, including progressive lymphopenia, absence of delayed-type hypersensitivity, and abnormal in vivo antibody production (Blaese et al., 1968; Cooper et al., 1968; Ochs et al., 1980; Sullivan et al., 1994). Most studies implicated a predominant T-cell defect (Gallego et al., 1997; Molina et al., 1993; Sullivan et al., 1994). This idea was reinforced by the discovery that WAS protein (WASp), the protein mutated in this syndrome, plays a crucial role in cytoskeletal remodeling downstream of T-cell receptor (TCR) engagement (Barda-Saad et al., 2005) and contributes to the immune synapse formation between T lymphocytes and antigen-presenting cells (Dupre et al., 2002). However, impaired maturation, decreased motility, reduced migration, and abnormal morphology, including absence of filopodia, have recently been recognized in B cells from WAS patients (Andreu et al., 2007; Park et al., 2005; Westerberg et al., 2005), and a unique role of WASp in marginal-zone B-cell homeostasis and function has been demonstrated (Meyer-Bahlburg et al., 2008; Westerberg et al., 2008). Abnormal natural killer (NK) cell function (Gismondi et al., 2004; Huang et al., 2005; Orange et al., 2002), defective invariant NKT cell (NKT) homeostasis and function (Astrakhan et al., 2009), and impaired T-cell migration and T-cell priming by dendritic cells (Binks et al., 1998; Bouma et al., 2007, 2009; de Noronha et al., 2005) indicate that innate immunity is also affected. In addition to microplatelet thrombocytopenia, characteristic functional and morphological abnormalities of WAS platelets were recognized (Gröttum et al., 1969; Kuramoto et al., 1970; Ochs et al., 1980; Semple et al., 1997). The findings of multiple food allergies, eosinophilia (Huntley and Dees, 1957), and elevated IgE levels suggest an allergic basis for the eczema (Berglund et al., 1968). An increased risk of malignancies, including lymphoma, malignant reticuloendotheliosis, and leukemia, was reported as early as 1961 (Imai et al., 2004; Kildeberg, 1961; Cotelingam et al., 1985; Sullivan et al., 1994; ten Bensel et al., 1966).

Distinguishable from the classic WAS phenotype (MIM 301000) is a milder form designated *hereditary X-linked thrombocytopenia* (XLT, MIM 313900) (Canales and Mauer, 1967; Chiaro et al., 1972; Jin et al., 2004; Notarangelo et al., 1991; Stormorken et al., 1991). In patients with XLT, eczema, if present, is mild; immune functions are less disturbed or normal, autoimmune disorders are rare, and malignancies appear to be less frequent (Albert et al., 2010). The thrombocytopenia may be present intermittently (Notarangelo et al., 2002).

The gene responsible for both WAS and XLT was initially mapped to Xp11.22 (Donner et al., 1988; Kwan et al., 1991). The gene, designated as *WAS*, was subsequently identified by positional cloning (Derry et al., 1994). *WAS* is composed of 12 exons containing 1,823 base pairs and encodes a 502-amino acid protein with a predicted molecular weight of 54 kDa. WASp is constitutively expressed in all hematopoietic stem cell-derived lineages and is located predominantly in

the cytoplasm, with the highest protein density being toward the cell membrane (Rivero-Lezcano et al., 1995; Zhu et al., 1997). WASp belongs to a family of cytoskeleton regulatory proteins that include N-WASp and the Scar/WAVE proteins 1–3 (Oda et al., 2005; Pollitt and Insall, 2009). Several functional domains based on the genomic structure of WASp have been identified, including the N-terminal WASp homology 1 (WH1) domain followed by a basic region (BR), a GTPase-binding domain (GBD), a polyproline-rich region, and a C-terminal Verprolin homology/Central region/Acid region (VCA) domain (Burns et al., 2004; Thrasher and Burns, 2010).

One of the essential roles of WASp is the regulation of actin polymerization by actin-related protein (Arp) 2/3, as illustrated by the finding that WAS macrophages fail to organize the Arp2/3 complex in podosomes (Linder et al., 1999). In this model, WASp is present in two configurations: the active form, in which the C terminus of WASp is free to interact with the Arp2/3 complex, and the inactive form, in which the C terminus forms an autoinhibitory contact with the BR of the GBD (Kim et al., 2000).

The distinct domains of WASp form the basis for its multiple functions, which include self-regulation, cytoplasmic signaling, and, through the interaction with Arp2/3, actin polymerization. The identification of the *WAS* gene has not only provided new insight into the critical role played by this complex molecule in cell movement and cell–cell interaction but also has had an impact on establishing a genotype–phenotype correlation (Albert et al., 2010; Imai et al., 2004; Jin et al., 2004; Lemahieu et al., 1999; Zhu et al., 1997), carrier detection, and prenatal diagnosis. Early diagnosis of WAS is crucial for optimal therapy, which includes antibiotics and intravenous immunoglobulin (IVIG) substitution, and hematopoietic stem cell transplantation and gene therapy to cure this devastating disease.

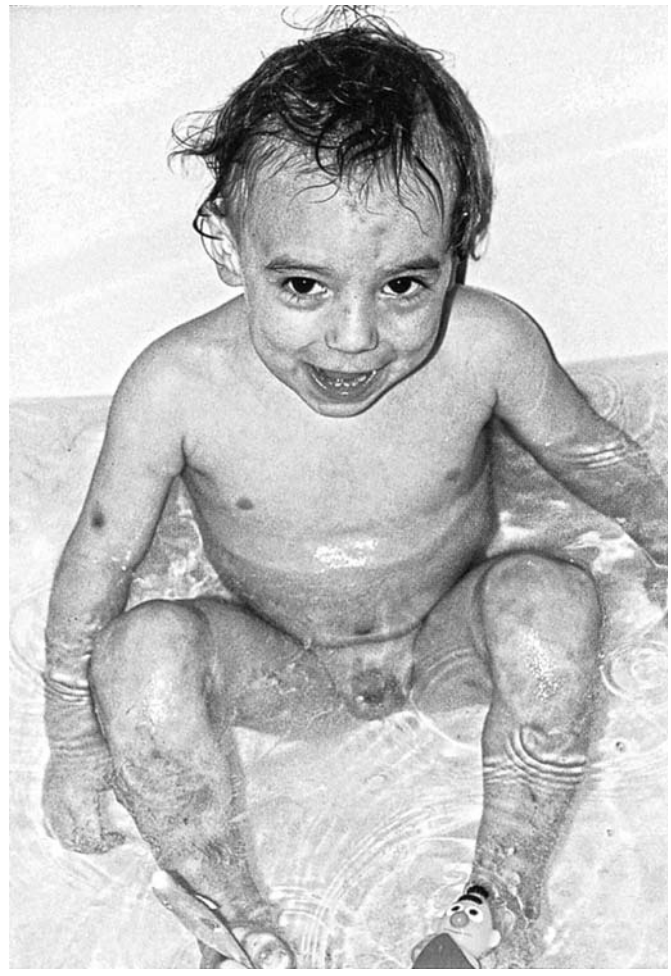

Figure 43.1 Color Plate 43.II Two-year-old WAS patient with a clinical score of 3. Note bruises and eczema of hands and legs. (See Color Plate.)

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

INCIDENCE, ONSET OF SYMPTOMS, AND AGE AT DIAGNOSIS

The incidence of the classic WAS phenotype has been estimated to be between 1 and 10 in 1 million individuals (Ryser et al., 1988; Stray-Pedersen et al., 2000). With broader awareness of the classic syndrome and the much milder XLT phenotype, along with the availability of reliable diagnostic tools, the true incidence is expected to be higher.

Early manifestations of WAS and XLT consist of petechiae, bruises, and bloody diarrhea and are often present at birth (Wiskott, 1937). Because of the increased risk of intracranial bleeding during vaginal delivery, birth by cesarean section is an option if the diagnosis is known prenatally. Excessive hemorrhage following circumcision is an early diagnostic clue. Eczema, either mild and localized or severe and generalized, is a consistent early manifestation of classic WAS (Fig. 43.1; Color Plate 43.II). The most characteristic findings at diagnosis, both in classic WAS and in XLT, are thrombocytopenia and small platelets. Infections, including otitis media with drainage of mucoid purulent material, are frequent complaints during the first 6 months of life. In a retrospective study of North American patients with a clinical presentation compatible with WAS/XLT completed before the *WAS* gene was identified, the average age at diagnosis was 21 months, with a range from birth to 25 years (Sullivan et al., 1994). The diagnosis was established earlier in patients with known affected family members (mean age at diagnosis 10 months) than in patients without a family history (24 months). Patients presenting with the XLT phenotype are often considered as having ITP, considerably delaying the actual diagnosis (Bryant and Watts, 2011).

INFECTIONS

Because of the profound cellular, humoral, and innate immune deficiency, infections are common manifestations of classic WAS. Upper and lower respiratory tract infections, often caused by common bacteria, are frequent and include otitis media (reported by 78 percent of WAS patients), sinusitis (24 percent), and pneumonia (45 percent) (Sullivan et al.,

1994). In the same retrospective study, sepsis was observed in 24 percent, meningitis in 7 percent, and "infectious" diarrhea in 13 percent. Severe viral infections do occur, including varicella with systemic complications that may require treatment with acyclovir and high-dose IVIG or varicella-zoster immunoglobulin (VZIG), and recurrent herpes simplex infections (observed in 12 percent). *Pneumocystis carinii (jiroveci)* pneumonia (PCP) (reported to affect 9 percent of WAS patients) is less prevalent than in patients with severe combined immune deficiency (SCID) or X-linked hyper-IgM syndrome (X-HIM) (Sullivan et al., 1994). Fungal infections, caused predominantly by *Candida* species, are relatively rare (10 percent of WAS patients) but may become more extensive during treatment with antibiotics. Patients with the XLT phenotype often lack a history of severe and frequent infections (Albert et al., 2010; de Saint Basile et al., 1996; Imai et al., 2004; Jin et al., 2004; Villa et al., 1995).

DEFECTS OF THE IMMUNE SYSTEM

Adaptive Immunity

The extent of the immune deficiency varies from family to family and depends largely on the mutation and its effect on WASp expression (Imai et al., 2004; Jin et al., 2004; Lutskiy et al., 2005a). Both T- and B-lymphocyte functions are affected. During infancy, the number of circulating lymphocytes may be normal or moderately decreased (Ochs et al., 1980; Park et al., 2004). By 6 years of age, lymphopenia due to loss of T lymphocytes is a common finding in patients with classic WAS (Ochs et al., 1980), and the numbers of B cells in lymph node follicles and T cells in the interfollicular area are reduced (Fig. 43.2; Color Plate 43.I). This may be in part due to accelerated cell death observed in peripheral blood lymphocytes from patients with WAS but not from those with XLT (Rawlings et al., 1999; Rengan et al., 2000). Abnormal T-cell function is suggested by diminished but not absent lymphocyte responses to mitogens (Cooper et al., 1968), depressed proliferative responses to allogenic cells (Ochs et al., 1980) and immobilized anti-CD3 monoclonal antibody (Molina et al., 1993), and complete lack of proliferation in response to periodate (Siminovitch et al., 1995). In a retrospective study of 154 patients, Sullivan et al. (1994) reported depressed proliferative responses to mitogens and allogenic cells in approximately 50 percent of the patients. Skin tests for delayed-type hypersensitivity were abnormal in 90 percent of the patients studied. Partial mixed chimerism due to the engraftment of only donor T lymphocytes seems to have corrected the immune defect, including antibody responses to polysaccharides, which suggests that the abnormal antibody production observed in WAS patients is largely caused by defective T-lymphocyte function (Parkman et al., 1978). The increased incidence of PCP also points to a significant T-cell defect in classic WAS. T cells from was$^{-/-}$ mice fail to spread, cap their TCR, proliferate, and secrete IL-2 in response to TCR cross-linking with anti-CD3 (Snapper et al., 1998).

Serum IgG and IgM levels are usually normal; IgA and IgE are frequently elevated. The fact that serum immunoglobulin

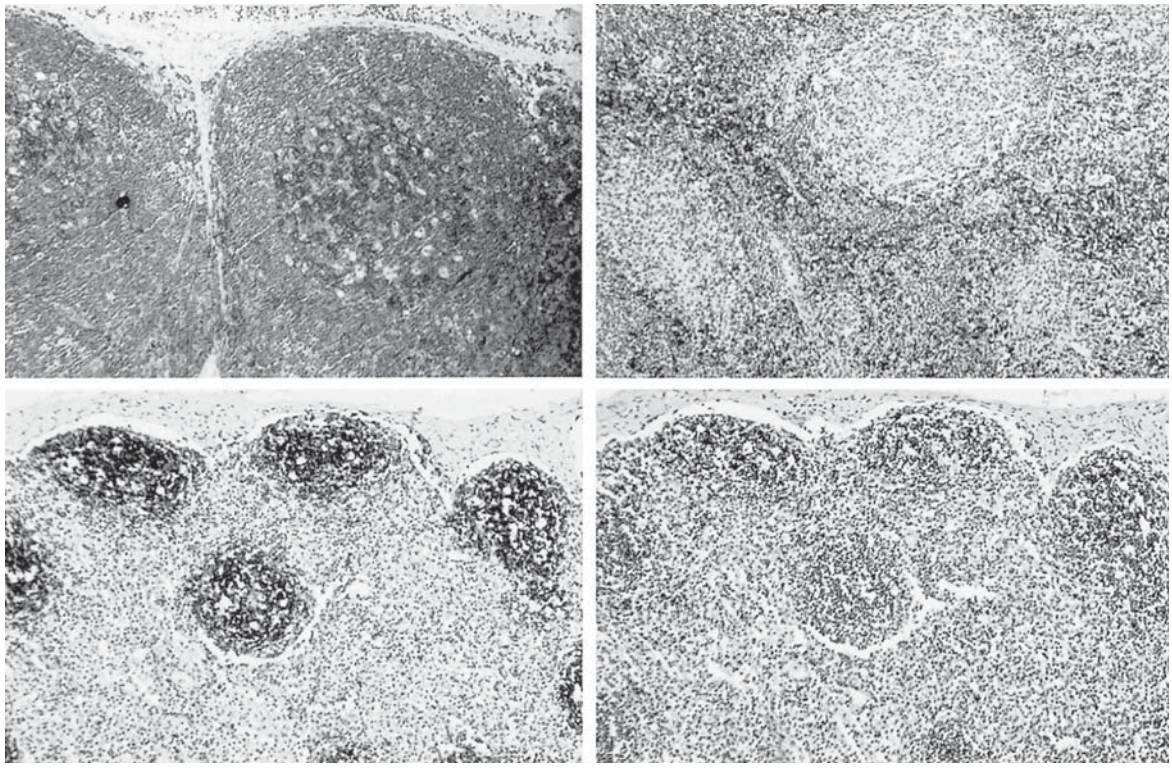

Figure 43.2 Color Plate 43.I Normal lymph node (*top*) and lymph node from a WAS patient (*bottom*). The left panels have been stained for B cells (in red). B cells are present in the poorly formed follicles of the WAS lymph node. The right panels have been stained for T cells (in red). The abundant numbers of T cells in the interfollicular area of the normal lymph node are sparse in the WAS lymph node (Perez-Atayde and Rosen, 1995). (See Color Plate.)

levels are normal or elevated suggests increased production, considering that the catabolism of immunoglobulin and albumin is twice that of normal controls (Blaese et al., 1971). The absolute number of B cells may be normal or moderately depressed (Park et al., 2005). Isohemagglutinin titers are frequently low (Ochs et al., 1980; Sullivan et al., 1994). Antibody responses are normal to some antigens and insufficient to others. A consistent finding is a markedly depressed response to polysaccharide antigens (Cooper et al., 1968; Ochs et al., 1980). This characteristic pattern of antibody deficiency is further illustrated by the observation that antibody responses to staphylococcal proteins are often normal, whereas antibody responses to streptococcal polysaccharides are consistently abnormal in WAS patients (Ayoub et al., 1968). In a multicenter retrospective review, antibody responses to a variety of protein antigens, including diphtheria and tetanus toxoid and to Hib vaccine (conjugated and unconjugated), were reported to be abnormal in more than 50 percent of WAS patients; in contrast, antibody responses to live virus vaccines were normal (Sullivan et al., 1994).

Antibody responses to intravenous immunization with bacteriophage ΦX174 are severely depressed in patients with classic WAS (who often lack WASp in lymphocytes and myeloid cells) and are characterized by absence of amplification and failure to switch from IgM to IgG (Fig. 43.3). These findings suggest abnormal class-switch recombination and lack of somatic hypermutation due to either defective T–B cell interaction or an intrinsic B-cell defect. In contrast, patients with the XLT phenotype mount a more robust response with amplification and isotype switching that may reach values comparable to those of normal individuals.

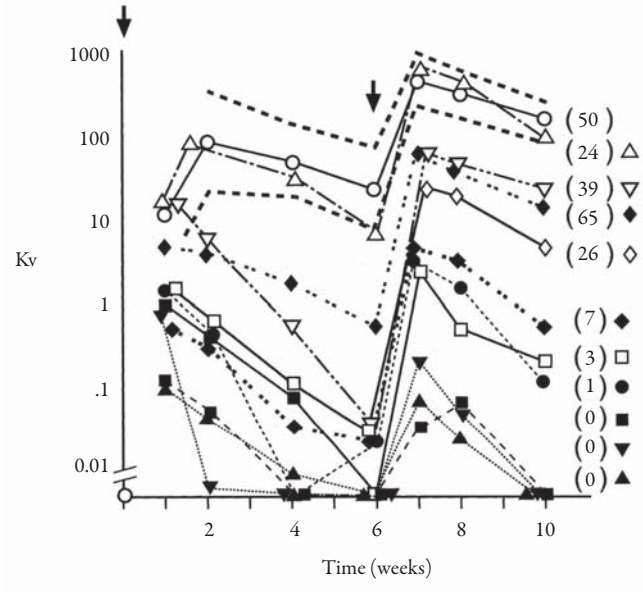

Figure 43.3 Antibody responses to the T-cell–dependent neoantigen, bacteriophage ϕX174 (phage), in patients with WAS/XLT. Phage was injected intravenously twice, 6 weeks apart (primary and secondary immunization), at a dose of 2×10^9 PFU/kg body weight (↓), and the production of phage-specific antibody was determined. Antibody titers were measured by a neutralizing assay expressed as rate of phage inactivation (Kv). The geometric mean antibody titers of normal males, following phage immunization, are indicated (○); ±1 SD is shown by broken lines. Percent IgG (in parentheses) was determined for serum samples obtained 2 weeks after secondary immunization by treatment with 2-mercaptoethanol (2-ME). Patients characterized by ♦, ◆ and ▲, ▼ are two pairs of cousins from two unrelated families with severe classic WAS (scores 4 and 5). The two patients indicated by the symbols ■ and ● are unrelated and also have classic WAS (score 3 and 4). The symbols Δ, ∇, □, ◊ indicate patients with XLT.

More recent studies suggest, however, that B-cell function is equally affected. Epstein-Barr virus (EBV)–transformed B lymphoblasts derived from WAS patients have reduced levels of filamentous (F) actin (Facchetti et al., 1998). B cells from WAS patients with mutations that lead to a complete absence of WASp, compared with normal B cells, have a defect in cell motility in response to CXCL13, and decreased ability to adhere homotypically and form long cytoplasmic protrusions after stimulation with anti-CD40 and interleukin (IL)-4 (Westerberg et al., 2005). By extending these studies to wasp-deficient mice, a reduced and delayed antibody response not only to T-dependent but also to T-independent antigens was consistently observed, supporting an intrinsic functional B-cell defect. It is possible, however, that this B-cell deficiency is secondary to the abnormal migration and impaired homing of B cells, reduced germinal-center formation, and abnormal marginal-zone (naïve) B-cell homeostasis and function observed in *was* knockout mice (Andreu et al., 2007; Meyer-Bahlburg et al., 2008; Park et al., 2005; Westerberg et al., 2005, 2008). Additional experimental evidence is required to determine if the observed B-cell deficiency is at least in part due to defective macrophage/dendritic or T-cell function (Bouma et al., 2007, 2009).

Innate Immunity

The susceptibility of WAS patients to viral infections and to malignancies may be the consequence of cytolytic or cytotoxic dysfunction. In normal NK cells, WASp can easily be detected, together with F-actin, in the immunological synapse. NK cells from patients with classic WAS lack WASp and show a markedly reduced accumulation of F-actin in the immunological synapse. As a direct result, WAS patients have defective cytolytic NK cell function (Orange et al., 2002). These findings were confirmed and expanded in a subsequent study of NK-cell cytotoxicity in both classic WAS and XLT patients (Gismondi et al., 2004). Although the percentages of NK cells were normal or increased, NK-cell cytotoxicity was inhibited in all patients with classic WAS and in most patients carrying mutations associated with the XLT phenotype. This inhibition of NK cell-mediated cytotoxicity, both natural and antibody-mediated, was associated with a reduced ability of WAS/XLT-NK cells to form conjugates with susceptible target cells and to accumulate F-actin on binding. Interestingly, addition of IL-2 corrected the functional defects of NK cells in vitro by affecting their ability to bind to target cells and to accumulate F-actin (Gismondi et al., 2004).

WASp-deficient humans and mice have regulatory T (Treg) cells that fail to suppress effector cells in vitro and that are incapable of controlling autoimmunity in mouse models (Adriani et al., 2007; Humblet-Baron et al., 2007; Maillard

et al., 2007; Marangoni et al., 2009). While WASp does not seem to be required for the thymic generation of natural Treg cells, it appears to play a crucial role in peripheral homeostasis of these cells (Humblet-Baron et al., 2007).

The involvement of the cytoskeleton in cell migration, active phagocytosis, and cell trafficking of myeloid cells, macrophages, dendritic cells, and Langerhans cells makes these cells vulnerable to *WAS* gene mutations. A large number of studies, some performed more than 35 years ago (Altman et al., 1974), have investigated the functionality of WASp-deficient monocytes, macrophages, and dendritic cells. In both WAS and XLT patients and in *was*$^{-/-}$ mice, a substantial defect in cell trafficking, pathogen clearance, and uptake of particulate antigen has been observed. Formation of the actin-rich phagocytic cup, which depends on the presence of WASp (Tsuboi and Meerloo, 2007), and IgG-mediated apoptotic cell phagocytosis are impaired in WASp-deficient macrophages (Leverrier et al., 2001; Lorenzi et al., 2000). The requirement of continuous WASp activity for podosome formation and function (Dovas et al., 2009) explains the complete failure to assemble podosomes in monocytes, macrophages, dendritic cells, and osteoclasts lacking WASp, which results in a severe defect of cell adhesion and motility (Burns et al., 2004; Calle et al., 2004a; Linder et al., 1999). WASp-deficient macrophages have an abnormal, elongated shape, lack a clear lamellipodium front, and show impaired chemotactic responses (Badolato et al., 1998; Calle et al., 2004a). With the use of wasp-deficient mice (Calle et al., 2004b) or an in vitro system (Ma et al., 2008), an extensive inhibition of osteoclast sealing ring formation and bone resorption has been demonstrated. Abnormalities are likely caused by defective filopodia and lamellipodia formation, since filopodia are involved in sensing the immediate microenvironment of the cell. Transfection of full-length *WAS* cDNA into WASp-deficient macrophages restores chemotaxis in response to CSF-1 (Jones et al., 2002). The in vivo homing of dendritic cells to the T-cell zones of secondary lymphoid tissues is impaired kinetically and quantitatively in wasp-deficient mice (Bouma et al., 2007; de Noronha et al., 2005).

The number of circulating neutrophils, the efficiency of neutrophil migration into the tissue, phagocytosis, and bactericidal activity are normal in WAS/XLT. However, in vitro chemotaxis of WAS neutrophils in response to various chemoattractants is insufficient, an abnormality that is most pronounced in the early phase of chemotaxis (Ochs et al., 1980).

PLATELET ABNORMALITIES

The platelet defect, thrombocytopenia and small platelet volume, is a consistent finding in patients with mutations in the *WAS* gene. Platelet counts vary from patient to patient and within affected individuals and may be as low as 5,000/mm^3 or as high as 50,000/mm^3. Higher counts, observed transiently, are often associated with inflammation or bacterial infections (Oda et al., 1998). Two families with intermittent thrombocytopenia associated with unique amino acid substitutions in the *WAS* gene have been described (Notarangelo et al., 2002). In most patients, the mean platelet volume (MPV) is half that of normal control subjects (3.8–5.0 fl vs. 7.1–10.5 fl), resulting in a thrombocrit of approximately 0.01%, compared with a normal range of 0.14% to 0.31% (Ochs et al., 1980). After splenectomy, platelet counts and platelet volume increase but are still less than that of normal controls (Haddad et al., 1999; Litzman et al., 1996). Partial but significant recovery of platelet counts following splenectomy suggests that the development of thrombocytopenia is at least in part due to the uptake and destruction of platelets by macrophages in the spleen or other reticular endothelial organs of patients with WAS or XLT (Baldini, 1972; Gröttum et al., 1969; Murphy et al., 1972). This interpretation is supported by the observation that platelet and macrophages co-localize in spleen sections from WAS patients, a finding strongly suggesting that damaged or antibody-coated platelets are ingested by macrophages in the spleen (Shcherbina et al., 1999). Increased expression of phosphatidylserine (PS) on circulating platelets from WAS patients (Shcherbina et al., 1999) and the presence of opsonizing anti-platelet antibodies in approximately half of wasp-deficient mice (Marathe et al., 2009) have been suggested as possible causes of increased phagocytosis of platelets (Shcherbina et al., 1999). In a recently published retrospective analysis of data collected by the French Registry of patients with WAS/XLT, a specific subset of infants ≤ 2 years of age was identified with an extremely poor prognosis. In addition to autoimmunity, inflammation and malignancies, half of these infants presented with Severe Refractory Thrombocytopenia (SRT), suspected, but not proven, to have anti-platelet autoantibody (Mahlaoui et al., 2013), suggesting that autoimmune thrombocytopenia can be a complication in WAS/XLT, especially in the very young. An alternative explanation for the thrombocytopenia is a decrease in platelet production. This possibility was suggested by the observation that autologous platelet survival in WAS and XLT patients was only reduced to half (5 days ± 1.3 days SD) that of normal (Ochs et al., 1980). A persistent finding was a decrease in platelet turnover, which was approximately 30 percent of the value found in normal subjects, indicating a significant platelet-production defect. Because the marrow megakaryocyte mass is normal or increased in WAS and XLT patients (Haddad et al., 1999; Ochs et al., 1980), it has been suggested that ineffective thrombocytopoiesis is at least in part responsible for the low platelet count. The discrepancy between a normal to increased marrow substrate available for platelet production (megakaryocyte cytoplasmic mass) and the low rate at which platelets actually appear in the circulation (platelet turnover) characterizes the platelet production defect known as ineffective thrombocytopoiesis (Slichter and Harker, 1978). It was hypothesized that this abnormality was caused by a defect in platelet demarcation and platelet release from megakaryocytes, possibly a direct consequence of the interaction of WASp with the cytoskeleton. This mechanism is supported by the observation of Kajiwara et al. (1999), who reported that in semisolid culture assays, bone marrow CD34$^+$ cells from WAS patients failed to form efficiently megakaryocyte colonies and to mature fully into platelet-releasing megakaryocytes. Using a megakaryoblastic cell line (MEG-01), Miki et al. (1997) showed that WASp is indispensable for

actin filament reorganization to microvesicles during megakaryocyte differentiation. In contrast, Haddad et al. (1999), studying megakaryocytes derived in vitro from $CD34^+$ cells, found normal megakaryocyte differentiation and pro-platelet formation in both WAS and XLT patients. Interestingly, the platelets produced in vitro were of normal size, whereas peripheral blood platelets from the same patient exhibited an abnormally small size. Moreover, F-actin distribution was abnormal and filopodium formation deficient in cultured megakaryocytes from WAS patients. In spite of these abnormalities, megakaryocytes from WAS patients migrated normally in response to stroma-derived factor-1α (SDF-1α). At this point, it is unclear if transendothelial migration of megakaryocytes is important for enhanced platelet formation, or if movement of WAS megakaryocytes would be abnormal if other chemotactic active factors were used. The observation that WASp is tyrosine phosphorylated after stimulation of platelets by collagen (Oda et al., 1998) may be relevant to megakaryocyte migration since collagen seems to be involved in the interaction between megakaryocytes and endothelial cells. It is also possible that abnormal interaction between WAS megakaryocytes and endothelial cells through adhesion signals other than SDF-1α might have a direct impact on platelet formation. Although wasp-deficient mice show only moderate thrombocytopenia, significant abnormalities have been demonstrated in platelet production in the bone marrow, suggesting that WASp plays an important role in platelet biogenesis. In particular, megakaryocytes from *was*$^{-/-}$ mice shed platelets ectopically within the bone marrow space and display functional defects in response to fibrillar collagen I (the major matrix component of bone), including impaired migration to SDF-1α, loss of α2β1 integrin-mediated inhibition of pro-platelet formation, and virtual lack of actin-rich podosomes, that are normally induced by interaction between collagen I and its receptors GPVI or α2β1 integrin (Sabri et al., 2006).

Several investigators have reported abnormal structure, function, and metabolism of WAS platelets (Baldini 1972; Kuramoto et al., 1970). Because it is generally difficult to examine the function of platelets from thrombocytopenic patients, the data obtained may merely reflect the reduced size or damage of platelets. Platelet aggregation defects, as determined by abnormal responses to ADP, collagen, and epinephrine, have been reported (Gröttum et al., 1969; Kuramoto et al., 1970). These findings have been interpreted as platelet functional defects, but the severe thrombocytopenia compromises interpretation. One splenectomized XLT patient who had enough platelets for aggregation studies showed normal aggregation (Ochs et al., 1980). Tsuboi et al. have recently discovered that WASp binds to the calcium and integrin-binding (CIB) protein in platelets and that WASp altered by missense mutations affecting exons 1–3 shows lower affinity for CIB than wild-type WASp. This impaired complex formation between mutant WASp and CIB reduces αIIbβ3-mediated cell adhesion and causes defective platelet aggregation, possibly contributing to the hemorrhagic diathesis of WAS/XLT patients (Tsuboi et al., 2006). WASp deficiency in human and murine platelets decreases αIIbβ3-mediated integrin "outside-in" dependent responses initiated by fibrinogen when measured by platelet adherence and spreading (Shcherbina et al., 2010). The fact that the incidence of severe, life-threatening bleeding is relatively small, in spite of platelet numbers being as low as 5,000/mm^3, suggests that WAS/XLT platelets function adequately in vivo. Nevertheless, the pronounced bleeding tendency due to persistent thrombocytopenia is often the first sign of WAS or XLT and may be the only clinically relevant symptom in XLT patients. The presence of petechiae or prolonged bleeding after circumcision in newborns may alert the physician and lead to early diagnosis. In a retrospective study of a large cohort of WAS patients, the clinical manifestation of bleeding was present in 84 percent, consisting of petechiae and/or purpura (78 percent), hematemesis and melena (28 percent), epistaxis (16 percent), and oral bleeding (6 percent). Life-threatening bleeding, including oral, gastrointestinal, and intracranial hemorrhage, occurred in 30 percent of WAS patients, with intracranial hemorrhage being observed in only 2 percent (Sullivan et al., 1994). In a recent retrospective study involving 173 XLT patients (median age 11.5 years), serious bleeding episodes were reported in 13.9 percent, including 18 events of intracranial hemorrhage with five fatalities (Albert et al., 2010).

Carrier females with mutations leading to classic WAS in half of their male offspring have normal platelet numbers, size, function, and survival time. This finding is explained by the nonrandom X chromosome inactivation in T cells, B cells, and platelets (Fearon et al., 1988; Gealy et al., 1980; Greer et al., 1989; Puck et al., 1990). However, carrier females in families with a mild XLT phenotype may have random X chromosome inactivation in hematopoietic cells, with occasional skewing in favor of the X chromosome with the mutation. In these rare situations, carrier females may present with the XLT phenotype, which is often confused with ITP (Bryant and Watts, 2011; Inoue et al., 2002; Lutskiy et al., 2002; Zhu 2002).

ECZEMA AND OTHER ATOPIC MANIFESTATIONS

Eczema is another characteristic finding (Fig. 43.1; Color Plate 43.II) that differentiates WAS from ITP (Wiskott, 1937). The typical skin lesions resemble acute or chronic eczema in nature and distribution. A history of eczema, mild or severe, transient or consistent, was reported by 81 percent of WAS patients (Sullivan et al., 1994). In the most severe cases, eczema is resistant to therapy and may persist into adulthood. Molluscum contagiosum, herpes simplex, or bacterial infections may develop in areas of the skin affected with eczema, often posing a therapeutic challenge. Patients with the XLT phenotype have either mild and transient eczema or none at all (Albert et al., 2010; Imai et al., 2004; Jin et al., 2004). Bacterial antigens appear to play an important role in the persistence of chronic eczema, because the skin lesions of WAS patients often respond promptly to treatment with systemic antibiotics. It has been hypothesized that defective chemotaxis of dendritic and Langerhans cells may play a role in generating T cells responsible for the development of atopic diathesis involving the skin (Thrasher et al., 1998). This hypothesis is further supported by the observation that eczema disappears during

conditioning but before the infusion of donor hematopoietic stem cells has occurred. The eczema tends to be worse in families with atopic diathesis, a finding suggesting that other genes responsible for allergies may have a modifying effect.

Some WAS patients develop allergies to certain foods or drugs, and exposure to these antigens may result in anaphylactic shock.

AUTOIMMUNE MANIFESTATIONS

Autoimmune diseases are frequent, having been reported in 40 percent of a large cohort of WAS patients (Sullivan et al., 1994). The most common autoimmune manifestation observed has been hemolytic anemia, followed by vasculitis involving both small and large vessels (McCluggage et al., 1999; Merlini et al., 2009), renal disease, Henoch-Schönlein–like purpura, and inflammatory bowel disease. Other less frequent autoimmune diseases include neutropenia, dermatomyositis, recurrent angioedema, uveitis, and cerebral vasculitis. Infants with severe refractory thrombocytopenia were recently identified as a subset of a large cohort of WAS/XLT patients with poor prognosis and considered to represent autoimmune thrombocytopenia ((Mahlaoui N., et al., 2013). A comprehensive report from a single center in France of risk factors, clinical features, and outcome of autoimmune complications in WAS further underlines the importance of this problem (Dupuis-Girod et al., 2003). Of 55 WAS patients, 40 had at least one autoimmune or inflammatory complication. Autoimmune hemolytic anemia was detected in 20 patients with onset before the age of 5 years. Arthritis was present in 29 percent, neutropenia in 25 percent, vasculitis including cerebral vasculitis in 29 percent, inflammatory bowel disease in 9 percent, and renal disease in 3 percent. A high serum IgM concentration was a significant risk factor for the development of autoimmune disease or early death. Of 15 patients with high serum IgM levels, 14 developed autoimmune hemolytic anemia. In contrast, low serum IgM concentration (a frequently observed finding in WAS/XLT) was a marker of a favorable prognosis. In a recent study by the European Bone Marrow Transplantation/ESID Working Party, autoimmune diseases were observed in 21 of 97 long-term survivors following hematopoietic stem cell transplantation. Of those with partial (mixed) chimerism, the incidence was 67 percent compared with only 14 percent in those with full chimerism (Ozsahin et al., 2008).

The incidence of autoimmune diseases in XLT patients is substantially less frequent than in classic WAS (Albert et al., 2010; Sullivan et al., 1994). Autoimmune complications were reported by 21 of 173 XLT patients enrolled in a worldwide retrospective study, with autoimmune nephropathy and hemolytic anemia being the most frequent manifestations. IgA nephropathy, with or without Henoch-Schönlein purpura, often causing chronic renal failure requiring dialysis or renal transplantation, was a frequent complication in Japanese patients with the XLT phenotype (Becker-Herman et al., 2011; Albert et al., 2010; Imai et al., 2004).

Several mechanisms may contribute to the pathophysiology of autoimmunity in WAS, including impaired function of regulatory T cells, defective phagocytosis of apoptotic cells, reduced apoptosis of activated lymphocytes, impaired secretion of Fas ligand, and B-cell intrinsic abnormalities (Humblet-Baron et al., 2007; Leverrier et al., 2001; Maillard et al., 2007; Marangoni et al., 2009; Meyer-Bahlburg et al., 2008; Nikolov et al., 2010; Recher et al., 2012).

MALIGNANCIES

Malignancies can occur during childhood but are more frequent in adolescents and young adults with the classic WAS phenotype (Brand and Marinkovich, 1969; Cotelingam et al., 1985; Kildeberg 1961; Sullivan et al., 1994; ten Bensel et al., 1966). In a large North American cohort, malignancies were present in 13 percent (Sullivan et al., 1994), the average age at onset of malignancies being 9.5 years. Considering the increasing life expectancy, it is reasonable to assume that the incidence of malignancies will further increase as (untransplanted) WAS patients get older. The most frequent malignancy reported is lymphoma, usually an EBV-positive B-cell lymphoma, which suggests a direct relationship with a defective immune system. Only 3 of 21 tumors—1 glioma, 1 acoustic neuroma, and 1 testicular carcinoma—were not of lymphoreticular origin (Sullivan et al., 1994). WAS-associated malignancies have a poor prognosis, as illustrated by the fact that only 1 of the 21 patients who developed a malignancy was alive more than 2 years after establishing the diagnosis. Bone marrow transplantation was attempted in five WAS patients with malignancies, but none survived more than 6 months (Sullivan et al., 1994). The incidence of malignancies in patients with the XLT phenotype is unknown but is less than in classic WAS. In a retrospective analysis, 9 of 173 XLT patients (5.2 percent) developed malignancies (Albert et al., 2010). The majority (n = 5) were of lymphoid origin; other malignancies reported included spinalioma, seminoma, pancreatic cancer, and myelodysplastic syndrome. Six of the nine patients had died of malignancies at the time of analysis.

LABORATORY FINDINGS

BLOOD CELL ABNORMALITIES

The finding that WASp is expressed in $CD34^+$ stem cells (Wengler et al., 1995) is compatible with the observation that in WAS all hematopoietic stem cell-derived lineages of nucleated cells are functionally abnormal, including lymphocytes, regulatory T cells, NK and NKT cells, neutrophils, macrophages, dendritic cells, and platelets.

The most consistent laboratory abnormality for both WAS and XLT is thrombocytopenia, associated with small platelet size. The other two findings of the classic triad of thrombocytopenia, immunodeficiency, and eczema are often absent at the initial evaluation, even in those developing a classic WAS phenotype later in life (Sullivan et al., 1994). Laboratory evidence to establish the diagnosis is therefore of prime necessity. Moderately severe lymphopenia is a consistent finding in patients with classic WAS (Ochs et al., 1980) and may be present at an early age (Park et al., 2004).

Abnormal function of T and B lymphocytes, regulatory T cells, and NK cells is generally associated with classic WAS. Absence or a reduced quantity of WASp in lymphocytes of WAS/XLT patients is the best confirmatory test (short of mutation analysis), allowing rapid diagnosis by a simple flow-cytometric technique (Yamada et al., 1999).

Iron-deficiency anemia is common in infants and children with WAS/XLT because of the constant loss of red blood cells. This can be corrected with increased dietary intake of iron. Chronic infection may further impair the production of red blood cells. Autoimmune Coombs-positive hemolytic anemia is a frequent complication (Sullivan et al., 1994) and needs to be recognized early for proper therapy.

The thrombocytopenia and small platelet size are present at birth, are persistent, and as a rule do not respond to prednisone or high-dose IVIG. Platelet counts may vary and are usually between 10,000 and 40,000 platelets/mm^3. During infection or acute autoimmune disease, the number of platelets may temporarily increase, without increasing in size. Thrombocytopenia may be intermittent in patients with characteristic missense mutations (Notarangelo et al., 2002) or absent, as in boys with X-linked neutropenia due to mutations within the Cdc42-binding site (Ancliff et al., 2006; Beel et al., 2009; Burns et al., 2004; Devriendt et al., 2001; Moulding et al., 2007).

OTHER ABNORMALITIES

Patients with WAS respond to infections or to autoimmune diseases with increased sedimentation rates and elevated C-reactive protein (CRP). Vasculitis is a common complication affecting small and large arteries (Ilowite et al., 1986). An unexpected high frequency of aortic aneurysms, four affecting the thoracic and one the abdominal aorta, were observed in a cohort of 38 WAS patients during routine imaging procedures (Pellier et al., 2011). Deposits of IgA-containing immune complexes were found in purpuric skin lesions and in the small bowel of a WAS patient with a Henoch-Schönlein purpura–like vasculitis (H. D. Ochs, unpublished observation). Hemolytic anemia is often due to a warm autoantibody to red blood cell surface antigens that can be demonstrated by direct or indirect Coombs test.

RADIOLOGICAL FINDINGS

X-ray films may reveal chronic lung disease, sinusitis, or mastoiditis. Subperiosteal hemorrhage is seen occasionally. Arteriograms, CT scans, or ultrasounds are useful for the detection of aneurysms (McCluggage et al., 1999; Merlini et al., 2009; Pellier et al., 2011). Brain scans are used if cerebral hemorrhage is expected.

HISTOPATHOLOGY

The lymphoid tissue and thymus are prime targets for pathological changes in patients with *WAS* mutations, although the degree of involvement varies considerably. A gradual loss of cellular elements occurs in the thymus and lymphoid organs (Cooper et al., 1968). The pathological findings in lymph nodes and spleens from WAS patients consistently reveal depletion of small lymphocytes from T-cell areas, prominence of the reticulum cell stroma, the presence of atypical plasma cells with and without plasmacytosis, and extramedullary hematopoiesis (Snover et al., 1981). Progressive depletion of germinal centers is also observed (Fig. 43.2; Color Plate 43.I). In a study of spleens obtained from XLT and WAS patients undergoing splenectomy, a significant depletion of the white pulp was noticed. This depletion was not limited to the T cell-dependent area but also involved the B-cell compartment. Specifically, a remarkable depletion of the marginal zone (MZ) was observed in WAS patients, an abnormality that may be directly responsible for the defective antibody response to polysaccharide and selected protein antigens. There was a strong correlation between morphological abnormalities in the splenic tissue and the clinical severity of the disease (Vermi et al., 1999). Patients with severe disease (scores of 4 and 5) had more severe depletion of the white pulp, including the T-cell area, B-cell area, and MZ thickness, than patients with lower scores (2 or 3). The lymphoid tissues and follicles of the gastrointestinal (GI) tract are usually normal (Cooper et al., 1968), although GI lymphoid depletion may occur (Wolff, 1957).

A variable degree of thymic hypoplasia has been observed. Cooper et al. (1968) described normal thymic architecture, normal corticomedullary differentiation, and intact Hassall's corpuscles despite a small thymus. Wolff (1967) reported two patients with thymic pathology, one with thymic hypoplasia and the other with thymic atrophy. The surface of peripheral blood lymphocytes from WAS patients, when scanned by electron microscopy, is devoid of microvillous projections, compared with normal lymphocytes (Fig. 43.4; Kenney et al., 1986). However, other studies indicate that WASp deficiency allows an intact microvilli organization, strongly suggesting that WASp is dispensable for lymphocyte microvilli formation (Majstoravich et al., 2004). This finding is in keeping with the notion that WASp-mediated Arp2/3 activation results in the formation of a network of short-acting filaments bound in

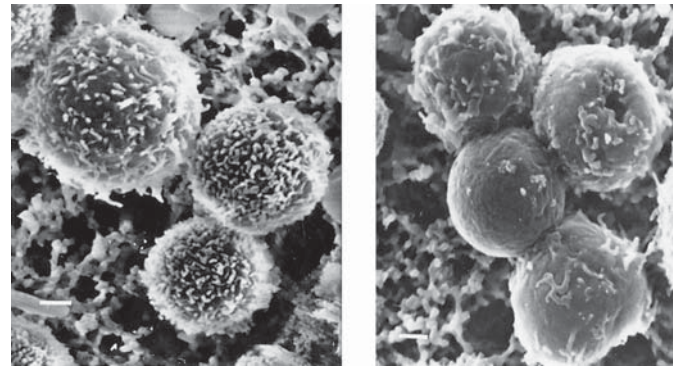

Figure 43.4 Scanning electron micrographs of normal T cells (*left*) and WAS T cells (*right*). The abundant microvilli covering the surface of the normal T cells are absent or sparse on the WAS T cells (from Kenney et al., 1986).

an end-to-side manner, as observed in macrophage and dendritic cell ruffles, whereas microvilli are composed of parallel bundles of side-to-side–linked actin filaments.

MOLECULAR BASIS

Through study of DNA from WAS families with multiple affected members, the *WAS* gene was mapped to the region Xp11.22-Xp11.3 (Kwan et al., 1991) and was then cloned by Derry et al. (1994), who demonstrated mutations in lymphoblastoid cell lines derived from WAS patients.

STRUCTURE OF WAS

The *WAS* gene consists of 12 exons spanning 9 kb of genomic DNA (Fig. 43.5). The 1,821 base pair cDNA generates a protein of 502 amino acids with a predicted molecular weight of 54 kDa. WASp is constitutively expressed in all hematopoietic stem cell-derived lineages and located predominantly in the cytoplasmic compartment, with the highest protein density being along the cell membrane (Rivero-Lezcano et al., 1995; Zhu et al., 1997). WASp is a key member of a family of proteins that link signaling pathways to actin cytoskeleton reorganization by activating Arp2/3-mediated nucleation of branched actin filaments. Several functional domains based on the genomic structure of WASp have been identified (Fig. 43.5), including the N-terminal WASp homology 1 (WH1) domain, a basic region (BR), a GTPase binding domain (GBD)/Cdc42 Rac Interactive Binding (CRIB) motif, a poly-proline region (PPR), a verprolin (V) homology domain, a cofilin (C) homology domain, and a C-terminal acidic (A) region (VCA) (Burns et al., 2004; Miki et al., 1996; Symons et al., 1996; Thrasher, 2002; Thrasher and Burns, 2010).

WASP HOMOLOGY 1 DOMAIN

The WH1 domain (also referred to as EVH1, Ena/VASP homology domain 1) is located close to the N-terminal region of WASp and N-WASp (a broadly expressed homolog of WASp). It includes a pleckstrin homology domain that is considered important for intracellular localization of WASp, through interactions with other proteins or lipids (Lemmon et al., 1996). Some data suggest that phosphatidylinositol 4,5 biphosphate (PIP_2) binds to the WH1 domain of WASp and N-WASp, resulting in PIP_2-dependent actin polymerization (Miki et al., 1996), but other observations indicate that PIP2 binds to the BR domain (Prehoda et al., 2000; Rohatgi et al.,

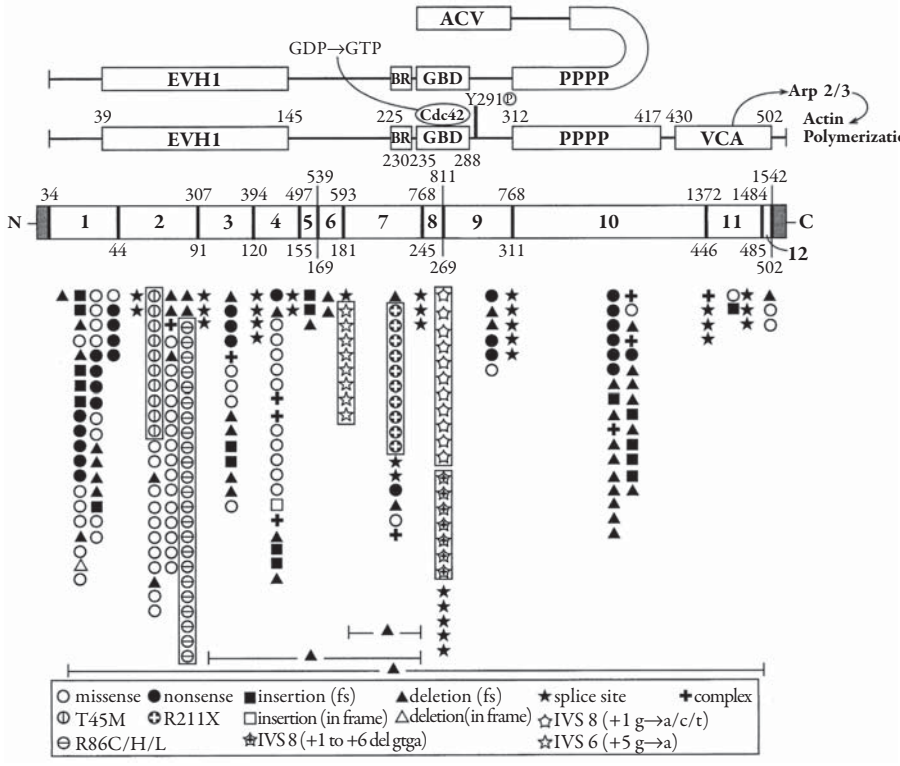

Figure 43.5 Schematic representation of the *WAS* gene, consisting of 12 exons (*center*). The top panels illustrate the major functional WASp domains and autoinhibition of WASp. The VCA domain interacts with a region from residues 242 to 310, which include the C-terminal part of the GBD (Kim et al., 2000). If Cdc42 is activated (GDP → GTP), WASP assumes the active form, allowing the C-terminal VCA domain to freely interact with Arp2/3 to initiate actin polymerization (see text for details). The *WAS* gene mutations identified in WAS families cared for in three centers (Seattle, Brescia, and Tokyo) are visualized according to their location in the exons and the exon–intron junctions. Each symbol represents a single WAS family. Missense mutations are located mostly in exons 1–4; deletions and insertions are distributed throughout the *WAS* gene; and splice-site mutations are found predominantly in introns 6, 8, 9, and 10. Arp2/3, actin-related protein 2/3; Br, basic region; EVH1, Ena/VASP homology 1 domain; GBD, GTPase binding domain; PPPP, proline-rich region; VCA, verpolin/cofilin homology domains/acidic region.

2000). There is evidence that PIP_2 synergizes with Cdc42 to fully activate WASp/N-WASp in vitro (Higgs and Pollard, 2000; Rohatgi et al., 2000).

Furthermore, the WH1 domain of WASp allows interaction with the WASp-interacting protein (WIP) (Ramesh et al., 1997). In resting lymphocytes, WASp/N-WASp constitutively associates with WIP, stabilizing WASp in its inactive conformation (Martinez-Quiles et al., 2001; Sasahara et al., 2002; Volkman et al., 2002). In keeping with this, WIP knockout mice show markedly reduced cellular levels of WASp. This defect can be partially reversed by treatment with calpain or proteasome inhibitors, indicating that WIP acts as a WASp chaperone by protecting it from protease- and proteasome-mediated degradation (Chou et al., 2006; de la Fuente et al., 2007). Missense mutations in the WH1 domain of WASp that impair interaction with WIP result in reduced levels of WASp without affecting its ability to bind Arp2/3 and to induce actin polymerization (Ramesh and Geha, 2009). These mutations in humans are most often associated with XLT (Imai et al., 2003). Following engagement of the T-cell antigen receptor, WIP also binds to the adaptor Crkl, which is part of a multimolecular complex that includes Crkl, WIP, and WASp, and is recruited by ZAP-70 to lipid rafts and the immunological synapse. TCR ligation causes protein kinase Cθ (PKCθ)-dependent WIP phosphorylation and disengagement of WASp from the WIP/WASp complex, thus allowing WASp activation by Cdc42 (Moreau et al., 2000; Sasahara et al., 2002). This leads to actin polymerization and stabilization of actin filaments (Volkman et al., 2002). The importance of WIP for stabilization of WASp was illustrated by a WIP-deficient patient due to a homozygous nonsense mutation in the *WIPF1* gene that resulted in lack of both WIP and WASp in patient leukocytes (vide infra) (Lanzi et al., 2012).

GTPASE-BINDING DOMAIN

The GBD is encoded by exons 7 and 8 of the *WAS* gene (Abdul-Manan et al., 1999; Aspenström et al., 1996; Kolluri et al., 1996; Symons et al., 1996) and allows interaction with Cdc42, a member of the Rho family of GTPases that regulates the formation of filopodia and controls cell polarity and chemotaxis (Hall, 1998). Like other proteins containing a GBD motif, WASp recognizes the GTP-bound but not the GDP-bound form of Cdc42 and binds to Cdc42-GTP with a 500-fold greater affinity than to Cdc42-GDP (Rudolph et al., 1998).

Computer modeling and binding experiments strongly suggest an autoinhibitory contact between the GBD and the carboxy-terminal region of WASp, which can be released by the activated (GTP) form of Cdc42 (Kim et al., 2000). Rho-like GTPases such as Cdc42 and Rac are key elements in the dynamic organization of the actin cytoskeleton (Lamarche et al., 1996). Thus, the GBD may have a direct effect on actin polymerization and an indirect effect on the interaction of the C terminus of WASp with the Arp2/3 actin nucleating complex (Fig. 43.5).

Toca-1 protein (transducer of Cdc42-dependent actin assembly) has been recognized as a crucial intermediate required for Cdc42/N-WASp/Arp2/3 complex-induced actin polymerization (Ho et al., 2004). To mediate Cdc42-induced activation of purified N-WASp by Toca-1, N-WASp must be complexed with WIP, thus demonstrating the importance of the WASp/N-WASp-WIP interaction in the regulation of actin polymerization.

POLY-PROLINE-RICH DOMAIN

A proline-rich region encoded by exon 10 contains the PXXP binding consensus for SH3 binding domains. WASp was shown to interact with SH3 domains of selected signaling molecules, including the cytosolic adaptor prtoeins, Grb2, $p47^{nck}$, Fyn, cFgr, Lck, c-Src, $p47^{phox}$, and proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1). WASp also interacts with the Tec family cytoplasmic tyrosin kinases, Btk, Tec, PLC-γ1, and Itk. This interaction may influence the localization of WASp and contribute to the conformational changes of WASp by regulating its phosphorylation. These observations suggest that WASp, through its interaction with the SH3 domain of multiple but selected molecules, plays an important role in cytoplasmic signaling of hematopoietic cells (reviewed in Imai et al., 2003). The proline-rich region is also required for the optimal actin polymerization activity of WASp (Castellano et al., 2001; Yarar et al., 2002) and for the recruitment of WASp to the immune synapse formed between T cells and antigen-presenting cells (Badour et al., 2003; Barda-Saad et al., 2005; Cannon et al., 2001).

VERPROLIN/COFILIN/ACIDIC REGION DOMAIN

The VCA domain, located in the C-terminus of WASp, plays a key role in the regulation of actin polymerization (Miki et al., 1996; Notarangelo and Ochs, 2003; Padrick and Rosen, 2010; Snapper and Rosen, 2003). If WASp is activated by GTP-Cdc42, the C-terminal region binds to Arp2 and Arp3 and to five unique polypeptides. WASp dimerization by SH3 domain proteins such as Nck, Grb2, Fyn, and PLC-γ1 has also been shown to increase the affinity of WASp for the Arp2/3 complex (Padrick et al., 2008). The actin monomer that binds to the VCA domain of WASp appears to be added to the Arp2/3 complex, thus promoting nucleation of the new daughter filament (Weaver et al., 2003).

FUNCTION AND REGULATION OF WASP

WASp, with its multifunctional domains, is responsible for key tasks of hematopoietic cells. The progress made over the past 10 years in understanding the biological functions of this complex protein has provided new insight into the pathogenesis and clinical presentation of WAS and XLT. WASp is expressed in $CD34^+$ hematopoietic precursors and in all lineage-committed cells. Carrier females of classic WAS show a nonrandom pattern of X chromosome inactivation in $CD34^+$ precursors and in all subsequent blood cell lineages (Wengler et al., 1995). This observation, along with evidence that hematopoietic stem cell (HSC) migration is defective

in *was*$^{+/-}$ mice (Lacout et al., 2003), suggested that WASp-deficient stem cells fail to transit successfully from the fetal liver to the bone marrow. However, more recent data have shown that the HSC compartment of *was*$^{+/-}$ mice contains an equal proportion of WASp-positive and WASp-negative cells. Progressive selection in favor of WASp-expressing cells is observed during lymphoid differentiation, especially in more mature cells (Meyer-Bahlburg et al., 2008; Westerberg et al., 2010). Furthermore, carrier females of XLT often show a residual proportion of WASp-negative cells, especially in the myeloid compartment. Taken together, these data indicate that WASp plays an important role especially at later stages in hematopoietic and lymphoid differentiation, and that this effect is more prominent in the lymphoid than in the myeloid compartment.

CYTOSKELETON AND ACTIN POLYMERIZATION

WASp is a key member of a family of proteins that link signaling pathways to actin cytoskeleton reorganization by activating Arp2/3-mediated actin polymerization (Takenawa and Suetsugu, 2007). This concept was suggested by Facchetti et al. (1998), who observed that the distribution of F-actin in EBV-transformed B lymphoblastoid cell lines from patients with classic WAS who lacked WASp was markedly reduced or completely absent compared with XLT subjects, who expressed reduced amounts of mutated WASp, and normal controls. In addition, cytoplasmic projections containing F-actin, recognizable as microvilli, were reduced in patients with WAS but not in patients with XLT.

Actin polymerization is initiated by the actin-related proteins (Arp), a group of proteins involved in the regulation of the actin cytoskeleton. WASp interacts directly with two of these proteins, Arp2 and Arp3, which form the Arp2/3 complex, leading to actin nucleation and formation of actin filaments (Carlier et al., 1999).

The binding of WASp to the Arp2/3 complex is mediated by the C-terminal acidic (A) region, which is preceded by the VC homology domain. This process is regulated by conformational changes in N-WASp (Rohatgi et al., 1999) and in WASp (Kim et al., 2000) by allosteric relief of autoinhibition through activation of Cdc42 and PIP_2 and by dimerization of WASp (Padrick et al., 2008). As discussed earlier, WASp is present in two configurations. In the active form, the C terminus of WASp is free to interact with the Arp2/3 complex; in the inactive form, the VCA domains interact with the hydrophobic core of the GBD. This binding is reinforced by the interaction of the acidic region (A) with a BR located N-terminal to the GBD (Kim et al., 2000). To revert to the active form, GTP-bound Cdc42 and PIP_2 cooperatively disrupt this autoinhibitory loop and release the C-terminal region for binding to the Arp2/3 complex (Fig. 43.5).

The importance of this self-inhibitory mechanism is exemplified by the observation that point mutations within the GBD of WASp can cause congenital X-linked neutropenia (XLN); these activating (gain-of-function) mutations will interfere with the autoinhibitory contact of the C terminus of WASp with the GBD, resulting in a permanently "active" configuration of WASp (Ancliff et al., 2006; Beel et al., 2009; Burns et al., 2004; Devriendt et al., 2001; Moulding et al., 2007). This unregulated activation of the actin cytoskeleton promotes hematopoietic cell death by enhanced apoptosis affecting mainly myeloid progenitors (Burns et al., 2004). Furthermore, lymphocytes from a patient with XLN (due to I294T mutation) displayed abnormal microvilli architecture and increased content of F-actin and failed to roll normally on L-selectin ligand under flow (Burns et al., 2010). Activating WASp mutations observed in XLN patients (L27OP; I294T) were studied for their effect on lymphocytes in knock-in mouse models generated by using RAG-2–deficient blastocyst complementation with target ES cells. Both activating WASp mutations led to enhanced actin polymerization, altered cytoskeletal responses, and genomic instability (Westerberg et al., 2010).

INTRACELLULAR SIGNALING

The proline-rich domain of WASp interacts with SH3 domains of selected cytoplasmic proteins, a finding suggesting that WASp is involved in intracellular signaling of hematopoietic cells. Phosphorylation by tyrosine kinases regulates signal transduction by connecting upstream cell-surface receptors to downstream pathways. WASp itself undergoes tyrosine phosphorylation following adherence of platelets to collagen (Oda et al., 1998). Baba et al. (1999) identified WASp as one of the major phosphoproteins associated with Btk. Together with Lyn and Btk, Hck has been shown to effectively mediate phosphorylation of WASp Tyr291 (Scott et al., 2002). Tyr291 is conserved in N-WASp and is positioned adjacent to the Cdc42 binding site. Phosphorylation of Tyr291 stabilizes the active conformation of WASp and exposes the VCA domain, allowing interaction with the Arp2/3 complex and enhancement of cytoskeletal reorganization, which is required for the formation of podosomes and filapodia (Cory et al., 2002). Dephosphorylation of Tyr291 by the tyrosine phosphatase PTP-PEST, by contrast, favors the formation of the autoinhibitory structure of WASp (Cote et al., 2002). To confirm the critical role played by Tyr291 phosphorylation, the homolog residue in mice (Tyr293) was replaced by Phe, thus preventing phosphorylation. Tyr293Phe knock-in mice develop significant immunodeficiency (Blundell et al., 2009). On the other hand, mice carrying the Tyr293Glu mutation (that mimics constitutive phosphorylation, as also observed in XLN patients with Tyr291Glu mutation) show reduced levels of WASp, which can be partially restored in vitro by proteasome inhibitors (Blundell et al., 2009). These data suggest that phosphorylation of Tyr291 may target WASp to proteasome-mediated degradation.

The proline-rich region of WASp allows binding to the SH3 domain of the linker protein, PSTPIP1, and may thus allow interaction with the CD2-associated protein CD2AP and recruitment of WASp to the immunological synapse (Badour et al., 2003).

T-cell antigen receptor engagement is crucial for the cytoplasmic signaling events that lead to cytoskeletal reorganization

through actin polymerization. This process is essential for cellular shape change, cellular movement, and immune synapse formation. Recruitment of WASp to the site of actin polymerization depends on a series of biochemical events that follow TCR engagement. In mature T lymphocytes, activation of the TCR results in the phosphorylation of multiple tyrosine residues of LAT (linker for activation of T cells) and SLP-76, which leads to the migration of Nck and WASp to the cell periphery, where these molecules accumulate at an actin-rich circumferential ring. This process ensures that the actin polymerization machinery is carried to the plasma membrane in the vicinity of the activated TCR. These events have been confirmed by direct visualization of the dynamic complexity of molecular recruitment, molecular interactions, and actin polymerization in single living cells in time and space (Barda-Saad et al., 2005). Impaired intracellular signaling is responsible for defective nuclear translocation of NFAT and reduced secretion of IL-2 upon in vitro activation of WASp-deficient T lymphocytes (Cianferoni et al., 2005).

In the absence of WASp, impaired TCR-mediated activation in response to glycolipid antigens affects also the function of invariant NK T (iNKT) lymphocytes, with reduced secretion of IL-4 and IFN-γ (Astrakhan et al., 2009; Locci et al., 2009).

WAS mutations also affect intracellular signaling and cytolytic activity of NK cells. Various mechanisms account for these defects, including poor immune synapse formation between NK lymphocytes and target cells, impaired signaling through CD16 and the activating receptor NKp46, and defective activation of NK cells following interaction with dendritic cells (Borg et al., 2004; Gismondi et al., 2004; Orange et al., 2002).

Reduced toxicity by CTL lines, established from WAS patients, against tumor B-cell lines may contribute to the development of hematological malignancies that are common in WAS (De Meester et al., 2010). Although WAS CTLs expressed normal levels of lytic molecules, the lytic granules appeared not to fully polarize toward the center of the CTL–tumor target cell contact area, compatible with defective synapse formation.

Finally, WASp participates also in integrin- and B-cell receptor (BCR)–mediated signaling of B lymphocytes, and defective WASp signaling may contribute to impaired retention of the MZ B cells (Meyer-Bahlburg et al., 2008) and to defective B-cell activation.

WASP PLAYS A ROLE IN TRANSCRIPTIONAL REGULATION OF TH1 IMMUNITY

Defective TH1 cytokine production by TH lymphocytes from WAS patients is associated with defective induction of the "TH1 master regulator," T-BET (Trifari et al., 2006). An explanation for this abnormality was recently proposed by Taylor et al., who provided evidence that WASp locates to the nucleus in normal lymphocytes, where it engages many TH-specific immune function genes under TH1-differentiating conditions (Taylor et al., 2010). WASp was found in differentiating TH1 cells at the proximal promoter locus of the *TBX21* gene, which encodes the transcription factor T-BET, as part of two distinct histone-modifying complexes (H3K4 trimethyltransferase and H3K9 trimethylase). This epigenetic molecular mechanism to regulate T-BET expression is severely impaired in TH1 cells from patients with classic WAS who lack WASp. In contrast, XLT patients with missense mutations that allow expression of mutated WASp display TBX21 dynamics that are similar to those in normal TH1 cells

CHEMOTAXIS AND PHAGOCYTOSIS

Podosomes are highly dynamic adhesion structures that are mainly found in monocytes, macrophages, osteoclasts, and dendritic cells (Monypenny et al., 2011). WAS macrophages are completely devoid of podosomes (Linder et al., 1999), resulting in defective adhesion and orientation in a chemotactic gradient (Badolato et al., 1998). Wasp-deficient murine dendritic cells fail to establish a leading edge, show defective attachment and detachment on fibronectin-coated surfaces, and display lack of chemokinesis to the chemokine CCL21 (de Noronha et al., 2005). Consequently, migration of Langerhans cells from the skin to the draining lymph node (Snapper et al., 2005) and accumulation of dendritic cells in the T-cell area of the spleen following immunization are impaired in wasp-deficient mice. Along with abnormal formation of the immunological synapse between dendritic cells and T lymphocytes (Pulecio et al., 2008), the impaired migration of dendritic cells may play a role in defective T-cell priming (Bouma et al., 2007).

Involvement of WASp in IgG-mediated phagocytosis has been demonstrated by the observation that this FcγR-dependent process is impaired in WASp-deficient peripheral blood macrophages (Lorenzi et al., 2000). In normal macrophages, WASp itself is actively recruited to the "actin cup"; in WASp-deficient macrophages, formation of the actin cup and local recruitment of tyrosine-phosphorylated proteins are markedly reduced. These findings suggest that the cytoskeletal structure responsible for phagocytosis is dependent on WASp expression. Clearance of apoptotic cells by macrophages and dendritic cells requires recruitment of WASp to the phagocytic cup. Lack of WASp results in delayed phagocytosis, both in vitro and in vivo (Leverrier et al., 2001). Interestingly, phagocytosis of particulate antigens is impaired also in macrophages from patients with X-linked neutropenia due to constitutive activation of WASp (Ancliff et al., 2006).

Reduced chemotaxis of WASp-deficient neutrophils was reported in WAS patients (Ochs et al., 1980) and in *was*$^{-/-}$ mice, both in vitro and in vivo (Snapper et al., 2005). Deficiency of WASp also leads to abnormal migration of T lymphocytes. *Was*$^{-/-}$ murine T cells respond with reduced migration when exposed to the chemokine CCL19, leading to impaired homing of T cells into the Peyer's patches (Snapper et al., 2005). On the other hand, impaired response of *was*$^{-/-}$ MZ B cells to S1P1 may contribute to the abnormalities of the MZ associated with WASp deficiency (Meyer-Bahlburg et al., 2008; Westerberg et al., 2008). Finally, NK lymphocytes from patients with WAS or with XLT show reduced chemotaxis in response to CXCL12 and CX3CL1, with reduced adherence

to ICAM-1 and VCAM-1 (Stabile et al., 2010). In contrast, T and B lymphocytes from mice with activating wasp mutations show normal chemotactic responses, but impaired adhesion and spreading (Westerberg et al., 2010).

ACCELERATED APOPTOSIS

A correlation between actin cytoskeletal function, mutations of WASp, and programmed cell death has been suggested (Kothakota et al., 1997; Melamed and Gelfand, 1999; Rawlings et al., 1999; Rengan et al., 2000). The morphological changes that occur during apoptosis require actin cytoskeletal remodeling, a process necessary for the execution of programmed cell death. Defective actin polymerization may explain the accelerated in vitro cell death of lymphocytes and the progressive cellular and humoral immunodeficiency observed in individuals with classic WAS.

The precise role of WASp in apoptosis is not clearly defined. The downregulation of a cell survival pathway or, conversely, the upregulation of a cell death pathway in WAS lymphocytes has been considered. Accordingly, reduced Bcl-2 expression (Rawlings et al., 1999) and increased expression of caspase-3 and the cell death receptor CD95 (Fas) (Rengan et al., 2000) by WAS lymphocytes, compared with that in XLT and control lymphocytes, have been reported.

MUTATION ANALYSIS

The cloning and sequencing of the gene responsible for WAS and XLT have provided a powerful tool for confirming the diagnosis in affected males, identifying carrier females, and performing prenatal diagnosis. Techniques are available to screen peripheral blood lymphocytes, NK cells, monocytes, and platelets for the presence or absence of WASp, to estimate the quantity of WASp by Western blot analysis or flow cytometry, and to sequence genomic DNA and/or cDNA for mutations in the *WAS* gene. (The updated nomenclature is provided by the Human Genome Variation Society, www.hgvs.org, and by den Dunnen and Antonarakis, 2001.)

SPECTRUM OF WASP MUTATIONS

Following the discovery of the gene responsible for WAS and XLT (Derry et al., 1994), large series of *WAS* mutation analyses were reported from clinical centers throughout Europe, North America, and Asia (Albert et al., 2010; Gulacsy et al., 2011; Imai et al., 2004; Jin et al., 2004) (see WASPbase at: http://bioinf.uta.fi/WASbase). Figure 43.5 summarizes the results of sequencing studies computed from three centers (Imai et al., 2004; Jin et al., 2004). Participants in these studies included natives of North, Central, and South America, Western and Eastern Europe, the Middle East, Southeast Asia, and Japan. As expected, the incidence and type of mutations are similar in all parts of the globe. In this cohort of 270 families with a total of 312 patients, 158 unique *WAS* mutations where identified. As shown in Table 43.1, the most common mutations observed were missense mutations (n = 93 families), followed by splice-site mutations (n = 59), short deletions (n = 46), and nonsense mutations (n = 39). Insertions, complex mutations, and large deletions made up 12 percent of the mutations identified. Most deletions and insertions result in frameshift and early termination of transcription. In addition, unique missense mutations within the GBD/Cdc42 binding domain have been reported to cause X-linked neutropenia (XLN, MIM300299) with a clinical phenotype very different from classic WAS/XLT. XLN, a rare form of severe congenital neutropenia (Boztug and Klein, 2009), was originally described in a three-generation family with five affected members that had an Leu270Pro mutation in the GBD/Cdc42 binding domain of WASp (Devriendt et al., 2001). Subsequently, additional families with missense mutations in this domain were reported. Affected members of two families with an Ile294Thr mutation, one family with a Ser272Pro, and one with an Ile276Ser mutation had neutropenia and normal platelet counts but reduced numbers of NK cells and abnormal lymphocyte proliferation in response to anti-CD3 stimulation (Ancliff et al., 2006; Beel et al., 2009). It was suggested that these "gain-of-function" mutations result in profound reduction in neutrophil production and/or increased apoptosis, the latter being observed in cultured patient-derived bone marrow progenitors. These findings are consistent with an intrinsic disturbance of normal myeloid differentiation as a cause of the neutropenia (Ancliff et al., 2006; Moulding et al., 2007). Affected patients respond to treatment with G-CSF. However, two patients from the original Leu270Pro XLN kindred treated with G-CSF were recently reported as having developed a myelodysplastic syndrome and acute myelogenous leukemia, respectively, both with somatic mutations in the G-CSF receptor (*CSF3R*) gene and monosomy 7 in the leukemic cells (Beel and Vandenberghe, 2009).

As was observed in smaller series reported earlier and subsequently (Derry et al., 1994; Fillat et al., 2001; Greer et al., 1996; Itoh et al., 2000; Kwan et al., 1995; Lemahieu et al., 1999; Remold-O'Donnell et al., 1997; Schindelhauer et al., 1996; Schwartz et al., 1996; Schwarz 1996;; Villa et al., 1995; Wengler et al., 1995; Zhang et al., 2010; Zhu et al., 1995, 1997), the predominant mutations of WASp are amino acid substitutions, typically located in exons 1–4 (Fig. 43.5, Table 43.1). Only eight missense mutations, one each in exons 6, 9, 10, and 12 and two each in exons 7 and 11, were observed downstream of exon 4. One of those, Pro361Thr in exon 10, is the only missense mutation identified to date affecting a proline in the polyproline region of WASp. In addition, two unrelated families, both with the XLT phenotype, had a point mutation affecting the termination codon of exon 12 (503 X > S), resulting in the absence of WASp (Imai et al., 2004; Jin et al., 2004).

The second most common WASp mutations, splice-site alterations, occurred predominantly in the downstream half (introns 6–11) of the *WAS* gene, as has been reported by others (Lemahieu et al., 1999; Schwarz 1996; Wengler et al., 1995; Zhu et al., 1997). Of 31 unique splice-site mutations, 22 affected a donor site and 9 an acceptor site. Mutations involving variant splice sites resulted in multiple splicing

Table 43.1 DISTRIBUTION OF MUTATIONS IN 270 FAMILIES WITH WAS OR X-LINKED THROMBOCYTOPENIA

MUTATION TYPE	FAMILIES AFFECTED N (%)
Missense	93 (34.5)
Splice	59 (22)
Deletion	46 (17)
Nonsense	39 (14.5)
Insertion	19 (7)
Complex + large deletions	14 (5)
Total	270 (100)

products that often included small amounts of normal *WAS* cDNA. Insertions and short deletions typically involving less than 10 nucleotides resulted in most instances in frameshift and premature stop of translation. Complex mutations were rare, involving double missense mutations, point mutations followed by a deletion, or a combination of deletions and insertions. Four large deletions were observed, resulting in the loss of several exons. In one case, the entire WASp coding region was deleted. Of all deletions and insertions, 58 percent were due to "slippage" caused by the deletion or insertion of an extra nucleotide within a stretch of identical basis (Imai et al., 2004; Jin et al., 2004).

MUTATIONAL HOT SPOTS

In the 270 unrelated families studied (Imai et al., 2004; Jin et al., 2004), six mutational hot spots, defined as occurring in seven or more unrelated families (>2.5 percent), were identified (Table 43.2). Three were point mutations within the coding region; all three involved CpG dinucleotides (C > T or G > A) caused by methylation and deamination of a cytosine to a thymidine in a sense or antisense strand. The other three hot-spot mutations involved splice sites.

The 134 C > T mutation, found in 10 families, results in the substitution of threonine with methionine at position 45; the 256 C > N/257G > N mutations, observed in 23 unrelated families, result in the substitution of arginine at position 86 with either a serine, glycine, cystidine, histidine, or leucine. The 631 C > T mutation, which converts arginine at position 211 to a stop codon, was found in 10 families. The IVS 6 + 5 g > a (c.559+5G>A) mutation, present in eight unrelated families, results in both abnormal and normal splicing products. The IVS 8 + 1 g > n (c.777+1G>N) mutation, identified in 11 families, results in the deletion of exon 8, leading to frameshift and premature stop of translation. The IVS8 + 1 (c.777+1) to +6 del gtga, resulting in the deletion of exon 8, frameshift, and early termination, was found in seven unrelated families. These six mutations account for 25.6 percent of all families included in this study (Imai et al., 2004; Jin et al., 2004). Three of these six mutations (134 C > T, 256 C > N/257 G > N, and IVS 6 + 5 g > a) were consistently found in WASp-positive patients with a mild phenotype (XLT) and low score, whereas the three other mutations (631C > T, IVS 8 + 1g > n, and IVS 8 + 1 to + 6 del gtga) were predominantly WASp negative and had a high score ($p < 0.001$) (Table 43.2).

SPONTANEOUS REVERSION OF MUTATIONS

Somatic mosaicism due to spontaneous reversions of the causative mutations or second-site mutations that restore WASp expression were described as early as 1998 (Ariga et al., 1998, 2001; Boztug et al., 2007, 2008; Davis et al., 2008, 2010; Davis and Candotti, 2009; Du et al., 2006; Humblet-Baron et al., 2007; Lutskiy et al., 2005a, 2008; Stewart et al., 2007; Trifari et al., 2010; Wada et al., 2001, 2003;). In comparison to other primary immunodeficiency diseases, the occurrence of this phenomenon seems to be significantly more frequent in WAS patients. In most instances reversions were noticed in patients with the classic WAS phenotype and affected only mutations that lead to complete absence of the protein. These spontaneously occurring reversions result in leukocyte

Table 43.2 WASP HOT-SPOT MUTATIONS AND CLINICAL PHENOTYPE

MUTATION	AFFECTED FAMILIES (PATIENTS)	% OF TOTAL FAMILIES	PATIENTS WITH SCORES OF 1–2.5*	PATIENTS WITH SCORES OF 3–5
134C > T(T45M)	10 (11†)	3.7	10	1**
256C > N/257G>N (R86N)	23 (25)	8.5	23	2**
IVS6 + 5g > a, fs stop aa 190	8 (14)	3.0	11	3**
665C > T(R211X)	10 (11)	3.7	1	10
IVS8 + 1g > n, fs stop aa 246	11 (14 †)	4.1	5	7
IVS8 + 1 to + 6 del gtga, fs stop aa 246	7 (7)	2.6	1	6
Total	69 (82)	25.6	51	29

*A score between 2 and 3 is listed as a score of 2.5. Scores 1–2.5 are considered XLT; scores 3–5 represent the WAS phenotype.

†One patient with the T45M missense mutation and two patients with the IVS8 + 1g > a splice-site mutation could not be scored because of insufficient clinical data.

**Of the six patients with high scores, four developed autoimmune diseases and one died of lymphoma at age 44 years.

Three of the hot-spot mutations (T45M; R86N; IVS6 + 5g > a) are associated with low scores and three hot-spot mutations (R211X; IVS8 + 1g > n; IVS8 + 1 to + 6 del gtga) are associated with high scores ($p < 0.001$). fs, frameshift.

populations that express WASp at different degrees (Fig. 43.6; Color Plate 43.III). In 2006, an international party (Stewart et al., 2007) collected data demonstrating that approximately 11 percent of 272 WAS patients examined had significant reversions. WASp-expressing revertant peripheral blood lymphocytes were detected in infants as young as 3 months and in adult patients who were in their fourth decade of life (Davis et al., 2008; Stewart et al., 2007; Wada et al., 2003). All patients reported to date were found to have reversions within circulating lymphocytes, predominantly $CD8^+$ subsets (Boztug et al., 2008; Davis et al., 2008; Du et al., 2006; Stewart et al., 2007); in addition, some patients were found to have reversions in $CD4^+$ T cells (Boztug et al., 2008; Davis et al., 2008; Du et al., 2006; Lutskiy et al., 2008), NK cells (Boztug et al., 2008; Du et al., 2006; Lutskiy et al., 2005b), and rarely in B lymphocytes (Boztug et al., 2008; Lutskiy et al., 2008). Neither myeloid cells nor megakaryocytes (platelets) from WAS patients were ever found to have undergone a reversion. Interestingly, $WASp^+$ Tregs were found in vivo to exhibit a marked selective advantage in a classic WAS patient with a spontaneous revertant mutation (Humblet-Baron et al., 2007), strongly suggesting that altered Treg fitness likely explains the autoimmune features in human WAS. The development of significant populations of revertant cells in WAS patients involves at least two steps: (1) a spontaneous reversion of the causative mutation or a second-site mutation in the *WAS* gene sequence that restores expression of WASp and possibly its function and (2) the in vivo selection/enrichment for the revertant cells with partial or full restoration of WASp expression and biological function. The mechanism of this molecular reversion is unknown and may involve errors in DNA polymerase or DNA repair activity, mutation-prone sequences, or exposure to toxic environmental events. The observation that in some patients the reverted cells carried multiple different second-site mutations, resulting in different missense mutations, suggests that certain sequences in the *WAS* gene are specifically susceptible to reversion. One such patient carrying a C995G nonsense mutation (Arg321X) in exon 10 developed reversions that included synonymous and nonsynonymous changes within the nucleotide triplet harboring the original mutation and a variety of nucleotide substitutions and deletions at variable distances from the C995 position that restored WASp expression by splicing out the original mutation (Davis et al., 2008). Interestingly, two brothers with a nonsense mutation in exon 1 (c.C58T; p.Gln20X) were found to have $WASp^+$ cell populations among T and B lymphocytes and NK cells that carried multiple second-site mutations, resulting in different missense mutations with discordant distribution in both siblings (Boztug et al., 2008).

An unsolved question is whether the restored or altered WAS protein is functional. Some patients with reversions are judged to demonstrate clinical improvement with time (Boztug et al., 2008), whereas other investigators note that despite the high proportion of functional revertant T cells, the patients still suffer from severe infections and autoimmune disorders (Trifari et al., 2010). This lack of clinical improvement was observed in patients whose $WASp^+$ revertant T cells exhibited a diversified TCR Vβ distribution and normal responses to TCR stimulation by anti-CD3 (Trifari et al., 2010). Although it is unclear in which precursor cells the reversions originated, these observations suggest that gene therapy by inserting a normal gene into hematopoietic stem cells is likely to result in normalization of T- and NK-cell and possibly B- and Treg-cell function. These results

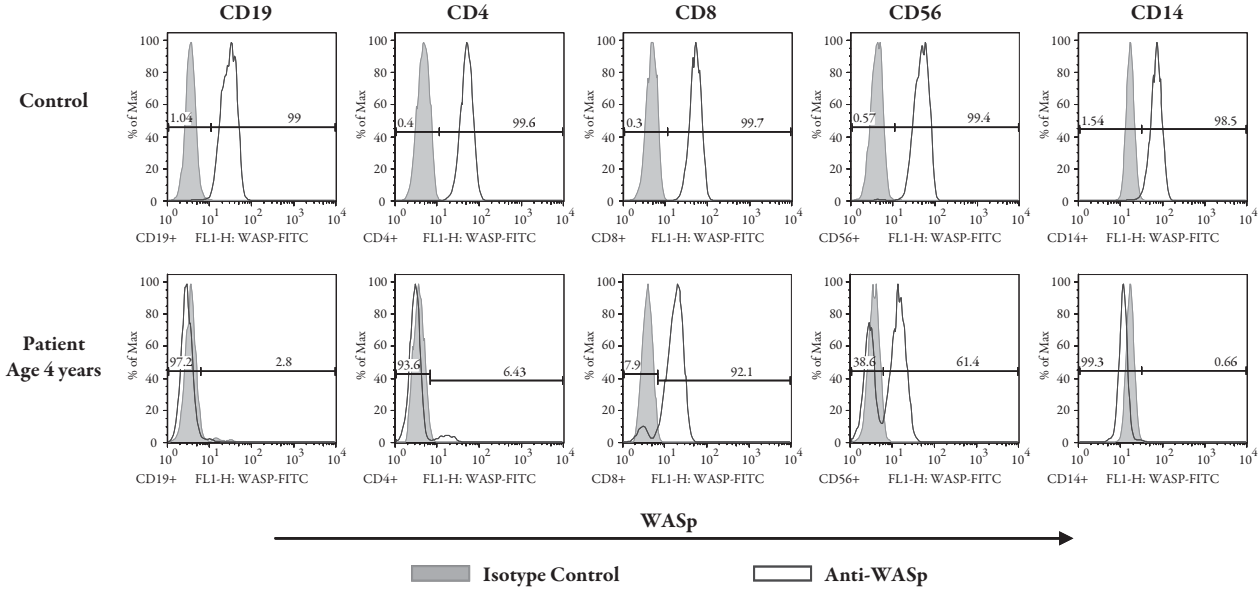

Figure 43.6 A WAS patient with a score of 2 (at 1 year of age), which changed to a score of 5 at age 2 years (due to vasculitis), had a nonsense mutation in exon 8 of the *WAS* gene (W252X) and, when studied at 1 year of age was, as expected, WASp negative. At age 4 years, he was found to have reverted his nonsense mutation to a missense mutation (W252S) that allowed expression of mutated WASp at normal intensity in most $CD8^+$ and $CD56^+$ cell populations and in a small proportion (6.4 percent) of his $CD4^+$ cells, but not in $CD19^+$ and $CD14^+$ lymphocytes, and not in platelets (*top panel* = control, *lower panel* = patient at 4 years of age). (See Color Plate.)

also underline the key role WASp plays in T-cell proliferation and in the in vivo survival and function of both T and NK cells and possibly B cells and Tregs.

GENOTYPE–PHENOTYPE CORRELATION

Mutations affecting the *WAS* gene result in three distinct phenotypes: (1) the classic WAS triad of thrombocytopenia and microplatelets, recurrent infections as a result of immunodeficiency, and eczema (Sullivan et al., 1994; Wiskott 1937), often complicated by autoimmune diseases and malignancies; (2) the milder XLT variant, characterized predominantly by thrombocytopenia and small platelets (Villa et al., 1995; Zhu et al., 1995), which can be intermittent (Notarangelo et al., 2002); and (3) congenital neutropenia without the clinical findings characteristic of WAS or XLT (Ancliff et al., 2006; Beel et al., 2009; Devriendt et al., 2001; Moulding et al., 2007). To clearly distinguish these clinical phenotypes, a scoring system, listed in Table 43.3, has been designed. The most consistent phenotype–genotype correlation was observed when the patients were divided into two categories: WASp positive if the mutated protein was present and of normal size and WASp negative if the protein was absent or truncated (Table 43.4). Patients with mutations that allow the expression of normal-sized mutated protein, often in reduced quantity, developed predominantly the XLT phenotype, whereas those patients whose lymphocytes could not express WASp or expressed only truncated WASp were more likely to have the WAS phenotype ($p < 0.001$) (Imai et al., 2004; Jin et al., 2004). When patients within one of the six hot spots were analyzed, there was a highly significant concurrence of the phenotype within each group (Table 43.2). Progression to a score of 5 due to either autoimmune disease or malignancy was observed in both groups but was far more frequent in WASp-negative patients with an initial score of 3 or 4 (Albert et al., 2010; Jin et al., 2004).

A retrospective analysis of 160 patients entered into the French WAS/XLT Registry identified 26 infants ≤ 2 years of age with a severe phenotype and poor prognosis. Of those 26 patients, half were categorized as severe refractory thrombocytopenia, with platelet counts persistently ≤ 10,000/μl, possibly due to anti-platelet autoantibody (Mahlaoui N., et al., 2013). Conceptually, this subgroup of infants characterized by early onset severe WAS with poor prognosis should be given a score of 5 and should undergo HSCT or gene therapy as soon as possible (Mahlaoui N., et al., 2013).

When the Japanese patients were analyzed separately (Imai et al., 2004), autoimmune diseases were equally frequent in patients with a low symptom score, representing XLT, and in those with a high score, representing WAS; this result was predominantly due to the high incidence of IgA nephropathy in the Japanese XLT patients. There were other exceptions to the rule, as is evident from data shown in Tables 43.2 and 43.4, a clear indication that it may be difficult in individual cases to accurately predict the clinical course based solely on the type of mutations in the *WAS* gene.

It is important to consider the complexity of the disease, the differences in lifestyle and medical care, chance exposure to unusual microorganisms, and the influence of genes that affect allergic predisposition, autoimmunity, and malignancies. Splice-site mutations, especially if affecting variant intronic nucleotide positions, often allow the generation of multiple splicing products, including the generation of normally spliced mRNA and the production of variable quantities of normal WASp. For example, the hot-spot mutation IVS6 + 5 g > a causes the insertion of 38 nucleotides from the proximal end of intron 6, which results in frameshift and early termination of transcription but also in the production

Table 43.3 SCORING SYSTEM* TO DEFINE PHENOTYPES OF *WAS* MUTATIONS

	XLN	IXLT	XLT		WAS (CLASSIC)		
Score	**0**	**<1**	**1**	**2**	**3**	**4**	**5**
Thrombocytopenia	–	–/+	+	+	+	+	+
Small platelets	–	+	+	+	+	+	+
Eczema	–	–	–	(+)	+	+/++	–/++
Immunodeficiency	–/(+)	–	–/(+)	(+)	+	+/++	–/++
Infections	+	–	–	(+)	+	+/++	–/++
Autoimmunity	–	–	–	–	–	–	+
Malignancy	+	–	–	–	–	–	+
Congenital neutropenia neutropenianeutropenia-nutropenia neutropenia	+	–	–	–	–	–	+
Myelodysplasia	+	–	–	–	–	–	–

XLN, X-linked neutropenia; IXLT, intermittent X-linked thrombocytopenia; XLT, X-linked thrombocytopenia.

*Scoring system: –/(+), absent or mild; –/+ intermittent thrombocytopenia; –, absent; (+), mild, transient eczema or mild, infrequent infections, not resulting in sequelae; +, persistent but therapy-responsive eczema or recurrent infections requiring antibiotics and often intravenous immunoglobulin prophylaxis; ++, eczema that is difficult to control or severe, life-threatening infections. Because patients with XLT may develop autoimmune disorders and/or malignancy, although at a lower rate than those with classic WAS, a progression from a score of 1 or 2 to a score of 5 is possible.

Modified from Stiehm ER, Ochs HD, Winkelstein JA, eds. *Immunologic Disorders in Infants and Children*, 5th ed. Philadelphia: Elsevier Saunders, 2004, with permission.

Table 43.4 EFFECT OF MUTATIONS ON EXPRESSION OF WASP

MUTATION TYPE	$WASP^+$ FAMILIES N (%)	$WASP^-$ FAMILIES N (%)
Missense	64 (73.6)	10 (8.9)*
Splice	12 [I = 3,V = 9] (13.8)	30 [I = 25, V = 5] (26.8)*
Complex	4 (4.6)	4 (3.6)
Deletion or insertion	5 (5.7)	32 (35.1)*
Nonsense	2 (2.3)	25 (22.3)*
Total†	87 (100)	112 (100)

I, invariant sites; V, variant sites.

*Distribution of mutations observed in $WASp^+$ and $WASp^-$ families is significantly different ($p < 0.001$). †From Imai et al., 2004; Jin et al., 2004; Not all patients were studied for expression of WASp.

of a small amount of normal *WAS* mRNA. Somatic mosaicism due to reversion is another possibility for a more favorable phenotype than that expected from the type of mutation (Boztug et al., 2008; Trifari et al., 2010; Wada et al., 2001). Genes that determine an individual's probability of developing atopic disease, such as allergies and eczema, or genes that control the effectiveness of host defense against infections may modify the WAS/XLT phenotype. Similarly, environmental factors, such as exposure to common or uncommon infectious agents, failure to establish the diagnosis at an early age, or less-than-optimal care, may affect the severity of the clinical presentation. Phenotype scoring before the age of 2 years is unreliable because it often, ironically, suggests a phenotype that is milder than expected from the type of *WAS* mutation identified (Jin et al., 2004).

STRATEGIES FOR DIAGNOSIS

Because of the wide spectrum of clinical findings, the diagnoses of WAS or XLT should be considered in any boy presenting with petechiae, bruises, and congenital or early-onset thrombocytopenia associated with small platelet size (Fig. 43.1, Color Plate 43.II). The presence of transient, mild, or severe eczema supports the diagnosis of XLT or WAS. It is important to note that infections and immunological abnormalities may be absent, mild, or severe, and autoimmune disease may develop more often in WAS than in XLT. Lymphopenia, characteristic for classic WAS, may be present during infancy but will develop invariably during childhood. Abnormal antibody response to bacteriophage ØX17 (Fig. 43.3) seems to be a consistent finding, even in patients with a mild phenotype. Screening for WASp mutations can be successfully performed by flow cytometry (Fig. 43.6; Color Plate 43.III), but patients with a substantial expression of mutated WASp may be missed. In atypical cases, mutation analysis of the *WAS* gene is essential for diagnosis; it may also assist in estimating the severity of the disease (with caution) and in performing carrier detection and prenatal diagnosis. It is vital to differentiate XLT from ITP patients (Bryant and Watts, 2011) because of the vulnerability of XLT patients to splenectomy, the need for different therapeutic approaches, and genetic implications.

FEMALES WITH WAS

Several symptomatic female patients have been identified as being heterozygous for mutations in the *WAS* gene. They presented with either a classic WAS phenotype (Parolini et al., 1998) or an XLT phenotype (Inoue et al., 2002; Lutskiy et al., 2002; Zhu 2002). In each case, the symptomatic females were found to have markedly skewed X-chromosome inactivation in favor of the X chromosome with the *WAS* mutation.

Sporadic cases of females with clinical and laboratory findings compatible with the diagnosis of WAS have been described (Conley et al., 1992; Evans and Holzel, 1970; Lin and Hsu, 1984). Two sisters, born to healthy parents, who presented with recurrent infections, thrombocytopenia, petechiae, and eczema had low serum IgM, normal IgG, and elevated IgA and IgE levels. They had decreased T-lymphocyte subsets and absent isohemagglutinins (Kondoh et al., 1995). Autosomal inheritance was also suspected in a second family with multiple affected female members, displaying clinical and laboratory findings resembling classic WAS (Rocca et al., 1996).

WIP DEFICIENCY—AN AUTOSOMAL RECESSIVE DISORDER WITH A WAS PHENOTYPE

A female infant with typical WAS symptoms and laboratory findings was found to have a mutation in *WIPF1* that encodes WASp-interacting protein (WIP) (Lanzi et al., 2012). The patient presented at 11 days of age with eczema, vesicular and ulcerative lesions of the skin and mucosa, poor weight gain, and thrombocytopenia (but with normal platelet volume). Family history was notable for a female sibling who had vesicular and ulcerative skin lesions and died of sepsis at 4 months of age. The patient's immune deficiency included defective chemotaxis, lack of T-cell proliferation in response to co-culture with anti-CD3, and abnormal NK-cell function. IgE was mildly elevated. Because of failure to thrive and chronic infections, she underwent hematopoietic stem cell transplantation at 4.5 months of age. Analysis of the *WIPF1* gene revealed a homozygous nonsense mutation in exon 6 (Ser434X) located upstream of the region encoding the WASp-binding domain

of WIP. Both parents, who are consanguineous, were heterozygous for this mutation. Both WIP and WASp were absent in the patient's leukocytes. This case illustrates that WAS cannot be diagnosed solely on the basis of lack of WASp expression but may require sequence analysis of *WAS*. WIP deficiency should be suspected in male and female patients with features of WAS in whom WASp is absent but WAS sequence and mRNA levels are normal. The diagnosis of WIP deficiency is confirmed by sequencing *WIPF1*.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

X-inactivation studies in WAS carrier females have shown that the normal X chromosome is preferentially used as the active X chromosome in all hematopoietic lineages, including $CD34^+$ cells (Wengler et al., 1995). Exceptions to this rule are families with milder forms of XLT, in which X chromosome inactivation may be random (Inoue et al., 2002; Lutskiy et al., 2002; Zhu, 2002).

If the *WAS* mutation is known in a given family, carrier females can be identified by mutation analysis. Similarly, prenatal diagnosis of a male fetus at risk for WAS or XLT can be performed by DNA analysis with chorionic villi sampling or cultured amniocytes as the source of genomic DNA (Giliani et al., 1999).

DIFFERENTIAL DIAGNOSIS

Several syndromes presenting with eczema, elevated serum IgE, and susceptibility to infections may resemble WAS/XLT. These include WIP deficiency (Lanzi et al., 2012); Omenn syndrome (see Chapter 13) due to hypomorphic mutations in genes associated with severe combined immunodeficiency (SCID), e.g. *RAG1/2*, *Artemis*, *ADA*, *IL-7R*, *IL-2Rγ*, *RMRP*; immune dysregulation, polyendocrinopathy, X-linked (IPEX, due to mutations in *FOXP3* [see Chapter 32]); Netherton syndrome due to mutations in *SPINK5* (Renner et al., 2009); hyper-IgE syndrome due to STAT3 or DOCK8 mutations (see Chapter 38); and atopic dermatitis.

ITP is a frequent misdiagnosis of patients with XLT. The fact that WAS/XLT platelets are consistently small and ITP platelets are large is useful for differentiating these two conditions. Automated platelet counting, unfortunately, does not pick up very small platelets, and the platelet size difference is less impressive.

The neutropenia associated with *WAS* gene mutations (XLN) is congenital and has to be differentiated from cyclic neutropenia due to *ELA2* mutations, and from severe congenital neutropenia due to *HAX1* mutation (Kostmann disease), *ELA2* mutations, *AP3B1* mutations (Hermansky-Pudlak syndrome) (see Chapter 51), and from WHIM (Warts, Hypogammaglobulinemia, Infections, Myelokathexis) syndrome due to mutations in the *CXCR4* gene (see Chapter 40), and from reticular dysgenesis due to mutations in the gene encoding mitochondrial adenylate kinase 2 (see Chapter 18).

TREATMENT

Progress in nutrition, improved antimicrobial therapy including antiviral and antifungal drugs, and the prophylactic use of IVIG have contributed to the prolonged life expectancy and improved quality of life of WAS patients. Early diagnosis is most important for effective prophylaxis and symptomatic and curative treatment (Albert et al., 2011). If an infection is suspected, careful evaluation for bacterial, viral, or fungal causes followed by appropriate antimicrobial therapy is of crucial importance. Infants with mutations in the *WAS* gene associated with lymphopenia are candidates for PCP prophylaxis. If antibody responses to protein and/or polysaccharide antigens are defective, which is most often the case in patients with the classic WAS phenotype, prophylactic IVIG infusions at intervals of 3 weeks at full therapeutic dose should be initiated. The dose of IVIG may have to be increased during infections, which may aggravate the already accelerated IgG catabolism. Continuous antibiotic therapy should be considered if infections occur despite IVIG therapy.

Killed vaccines can be given, including pneumococcal polysaccharides and protein-conjugated vaccines; the response, however, may be suboptimal and should be determined. Live-virus vaccines are not recommended for patients with the classic WAS phenotype. Varicella-zoster immunoglobulin or high-dose IVIG (which has high antivaricella antibody titers) and acyclovir are indicated following exposure to chickenpox. Antiviral drugs are also successfully used to treat recurrent herpes simplex infections.

Eczema, if severe, requires aggressive therapy including local steroids and, if indicated, short-term systemic use of steroids. Cutaneous infections are common and may necessitate local or systemic antibiotics. Lymphadenopathy is frequently observed and may be the consequence of infected eczema. Tacrolimus ointment (Protopic 0.03 percent) or pimecrolimus cream (Elidel 1 percent) are alternatives to steroid cream in the treatment of WAS-associated eczema. An appropriately restricted diet based on specific food allergies may have to be selected.

Platelet transfusions are reserved for serious bleeding—for example, central nervous system hemorrhages or GI bleeding. Frequent platelet transfusions may induce antibody responses to allogenic/autologous platelets, resulting in nonresponse to platelet transfusions and/or ITP. Blood products are best irradiated and should be cytomegalovirus negative. Aspirin, which interferes with platelet function, is contraindicated.

Splenectomy, sometimes recommended for XLT patients, effectively stops the bleeding tendency by increasing the number of circulating platelets. However, splenectomy increases the risk of potentially fatal septicemia and, if performed, requires lifelong antibiotic prophylaxis (Albert et al., 2010). Data collected at the National Institutes of Health support the clinical experience that splenectomy does not affect the development of autoimmune diseases or malignancies (M. Blaese, personal communication). High-dose IVIG, systemic steroids, or rituximab may be considered for the treatment of autoimmune complications. Long-term immunosuppressive therapy, however, may cause reactivation of viruses or increase the risk of fungal infections.

Because of poor dietary intake and constant loss of red cells, prophylactic treatment with iron is necessary to prevent chronic anemia.

TRANSPLANTATION

Hematopoietic stem cell transplantation, using as source bone marrow, peripheral blood, or cord blood, is the only curative therapy for WAS (Kobayashi et al., 2006; Moratto et al., 2011; Pai et al., 2006; Pai and Notarangelo, 2010). To prevent rejection, a conditioning regimen of busulfan in combination with cyclophosphamide, or fludaribine or antithymocyte globulin with or without irradiation is necessary (Kapoor et al., 1981; Moratto et al., 2011; Ochs et al., 1982; Parkman et al., 1978, Stepensky et al., 2013). Fully matched siblings are preferred donors and result in highly successful stem cell transplantation, even in older patients (Antoine et al., 2003; Filipovich et al., 2001; Moratto et al., 2011). Matched related or unrelated donors have been used in recent years with favorable outcome (Friedrich et al., 2009; Moratto et al., 2011; Pai and Notarangelo 2010). Cord blood stem cells, fully or partially matched, are excellent alternatives for boys with WAS who weigh less than 25 kg and are free of acute infections, if a fully matched bone marrow donor is not available (Bhattacharya et al., 2005; Jaing et al., 2007; Kaneko et al., 2003; Knutsen et al., 2003; Thomson et al., 2000). Haploidentical bone marrow transplants have had disappointing results and are generally not recommended (Antoine et al., 2003; Brochstein et al., 1991; Friedrich et al., 2009; Hong, 1996; Kobayashi et al., 2006; Mahlaoui et al., 2013; Ozsahin et al., 1996; Pai et al., 2006), although reports describing successful engraftment of unmanipulated HLA-haploidentical bone marrow from a parent, resulting in stable chimerism, have been published (Friedrich et al., 2009; Inagaki et al., 2005; Moratto et al., 2011).

Recent reports demonstrate the high rate of success of stem cell transplantation when HLA-identical related (e.g., siblings) or HLA-matched unrelated donors were selected (Friedrich et al., 2009; Moratto et al., 2011). Myeloablative but not "reduced-intensity" conditioning is required to achieve complete donor chimerism. The importance of this postulate is underscored by the recent report of a large European study indicating a strong association of posttransplant autoimmunity with a mixed/split chimerism status, strongly suggesting that residual host lymphocytes can initiate posttransplant autoimmune disease (Ozsahin et al., 2008).

GENE THERAPY

The demonstrated ability of hematopoietic cell transplantation to fully correct the disease has prompted several groups to explore novel therapeutic approaches to WAS based on gene transfer. In initial attempts, primary T and B lymphocytes, herpesvirus saimiri-immortalized cell lines, and dendritic cells derived from WAS patients have been successfully transduced with lentiviral vectors expressing the human *WAS* gene. Following transduction, the cells demonstrated a substantial growth advantage; responded to TCR signaling, including downregulation of CD3 surface expression; formed stable B- and T-cell conjugates; secreted normal amounts of IL-2 upon appropriate stimulation; and showed normal cytoskeletal reorganization (Charrier et al., 2007; Dupre et al., 2004; Martin et al., 2005; Miao, 2004). Furthermore, it has been demonstrated that targeting of human hematopoietic stem cells with a *WAS*-containing retroviral vector reconstitutes the actin cytoskeleton in myeloid progenitor cells that underwent in vitro differentiation (Dewey et al., 2006). These encouraging results have been fully replicated in *was*$^{-/-}$ mice that have been injected with *was*-lentiviral-corrected hematopoietic progenitor cells, with significant multilineage engraftment of gene-corrected cells, normalization of T-cell proliferation and IL-2 production, and improvement of the migratory defects (Astrakhan A. et al., 2012; Blundell et al., 2008; Charrier et al., 2005; Dupre et al., 2006; Marangoni et al., 2009). Experience with *was* gene transfer in mice has provided strong evidence for selective advantage of gene-corrected cells. This is reminiscent of what has been observed in WAS patients who develop somatic reversions that restored WASp expression and function (Boztug et al., 2007, 2008; Davis et al., 2008, 2010; Du et al., 2006; Humblet-Baron et al., 2007; Lutskiy et al., 2005a, 2008; Trifari et al., 2010; Wada et al., 2001). One important question that animal models cannot adequately address is whether gene therapy will correct the platelet defect.

Based on cellular and murine models, a clinical gene therapy protocol using transplantation of autologous hematopoietic stem cells transduced with a GALV pseudotyped MLV-derived retroviral vector was developed in Hannover, Germany. Initially, in 2006, two patients with mutations in the *WAS* gene were enrolled and autologous CD34^{+} hematopoietic stem cells were collected by leukapheresis and transduced with a WASp-expressing retroviral vector. Prior to infusion of the genetically modified hematopoietic progenitor cells, the patients underwent partial ablation with busulfan at a dose of 4 mg/kg of body weight per day on days −3 and −2. Except for transient myelosuppression and partial alopecia, no therapy-associated side effects were observed, and both patients showed expression of WASp in lymphoid and myeloid cells within 3 to 6 months after gene therapy (Boztug et al., 2010). In addition, sustained increases in platelet numbers, as well as in WASp^{+} thrombocytes, were observed. The clinical condition of both patients improved markedly, with resolution of bleeding tendency, eczema, autoimmunity, and predisposition to severe infection. Comprehensive insertion-site analysis showed vector integration that targeted multiple genes controlling growth and immunological responses in a persistently polyclonal hematopoiesis (Boztug et al., 2010). In 2009, this clinical gene therapy trial was expanded by including eight additional WAS patients. Overall, as of January 2012, 9 of 10 patients had evidence of sustained engraftment of WASp^{+} hematopoietic progenitor cells, and WASp expression was detected in 10 to 60 percent of myeloid cells and in most platelets, and had increased in T lymphocytes and NK cells to more than 80 percent 1 year following gene therapy. One patient with insufficient gene-treated cells failed to respond and underwent successful hematopoietic stem cell transplantation. Three patients developed a vector-associated acute T-cell

lymphocytic leukemia, two with integration of the vector in the *LMO2* gene region, and are undergoing chemotherapy. One of the three WAS patients with leukemia subsequently received a hematopoietic transplant (Christopher Klein, personal communication). Lentiviral-mediated transfer of the *WAS* gene (Merten et al., 2011) has recently been initiated in several gene therapy centers to achieve optimal transduction of hematopoietic stem cells and hopefully without vector-associated complications.

ANIMAL MODELS

Mouse models of wasp deficiency have been generated by gene knockout technology (Snapper et al., 1998; Zhang et al., 1999). wasp-deficient mice exhibit poor T-cell proliferation and cytokine secretion in response to TCR activation by cross-linking of CD3 and show mild lymphopenia, which can be normalized by transplantation (Strom et al., 2002). Although *was*$^{-/-}$ mice produce high titers of antinuclear antibodies, their susceptibility to frank autoimmune disease is controversial. In particular, vasculitis and nephritis have not been reported in was knockout mice. Furthermore, while virtually all *was*$^{-/-}$ mice on a Sv129 background develop severe colitis by 6 months of age (Nguyen et al., 2007), this is not observed in other backgrounds. *Was*$^{-/-}$ mice are at high risk of severe complications following challenge with *Streptococcus pneumoniae* (Andreansky et al., 2005), thus recapitulating the susceptibility of WAS patients to encapsulated pathogens. On the other hand, an important limitation of mouse models of WASp deficiency is the mild degree of thrombocytopenia, which does not lead to hemorrhagic manifestations.

Recently, wasp-activating mutations that correspond to those observed in patients with X-linked neutropenia have been introduced in murine embryonic stem cells that have been injected into a Rag2-deficient blastocyst. This has permitted researchers to confirm that constitutively active WASp interferes not only with myeloid development but also with T- and B-cell function, and leads to significant genomic instability (Westerberg et al., 2010). However, full characterization of the effect of these mutations will await development of a knock-in mouse model.

CONCLUSION AND FUTURE DIRECTIONS

The clinical phenotypes associated with mutations in the *WAS* gene are as complex as the function of its product. By studying WAS, XLT, and XLN patients and their specific *WAS* gene mutations, we have learned how a single gene defect can cause unique clinical symptoms. These investigations have provided new insight into the functions of WASp that facilitate not only cell–cell interaction and TCR-initiated cytoplasmic signaling but also cytoskeletal reorganization and actin polymerization, and neutrophil homeostasis. The structure of WASp is responsible for two configurations of the molecule—the active form, in which the C-terminus interacts with Arp2/3 to initiate actin polymerization, and the inactive form, in which the C-terminal forms an autoinhibitory contact with the GBD/Cdc42 binding domain. In the final analysis, WASp is required for efficient thrombocytopoiesis and platelet survival, T-cell activation, T- and B-cell interaction, NK cell function, Treg competitiveness, and effectiveness of the innate immune system. Since mutations of WASp interfere with the homing of hematopoietic stem cells, and thus affect all hematopoietic cell lineages, stem cell transplantation is the treatment of choice for curing this disease. For the same reason, gene therapy is expected to work, and this is one of the challenges to improve life expectancy and quality of life of affected patients.

Using conditional gene deletion to generate mice with selective deficiency of wasp in the B-cell lineage, severe abnormalities in the peripheral B-cell compartment occurred, including reduction of marginal-zone B cells and inability to respond to T-independent antigens. The resulting B-cell dysregulation, characterized by increased serum IgM levels, expansion of germinal-center B cells and plasma cells, and autoantibody production, suggests that wasp-dependent B-cell intrinsic mechanisms contribute to the increased incidence of autoimmunity observed in WAS (Recher et al., 2012).

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44.

X-LINKED LYMPHOPROLIFERATIVE DISEASES

Volker Schuster and Sylvain Latour

X-linked lymphoproliferative (XLP) disease is a rare immunodeficiency characterized by a peculiar susceptibility to Epstein-Barr virus (EBV) infection. Mutations in the gene *SH2D1A* coding for SLAM-associated protein (SAP) underlie 80 percent of familial XLP cases. In 2006, the remaining 20 percent of familial XLP cases were shown to harbor mutations in the gene *XIAP* (also termed *BIRC4*), and more recently, a few patients with impaired Mg^{++} flux resulting in impaired T-cell receptor (TCR) signaling caused by mutations in *MAGT1* were identified (Li et al., 2011). By several aspects, XLP can be lumped into the group of familial hemophagocytic lymphohistiocytosis (FHL) (Marsh et al., 2010a; Marsh and Filipovich, 2011) (see Chapter 53).

X-LINKED LYMPHOPROLIFERATIVE DISEASE DUE TO DEFECTS OF SH2D1A (SAP) (XLP-1)

X-linked lymphoproliferative disease type 1 (XLP-1; MIM #308240) is an inherited immunodeficiency involving primarily T, natural killer (NK), and natural killer T (NKT) cells that in most cases is exacerbated following exposure to EBV. In older children and adults, EBV, one of the eight known human herpesviruses, is the causative agent of infectious mononucleosis (Henle and Henle, 1979). This is usually a self-limiting polyclonal lymphoproliferative disease with an excellent prognosis. In immunosuppressed individuals, EBV infection may lead to life-threatening lymphoproliferative disorders and lymphoma (Hanto et al., 1985). Moreover, EBV has been associated with certain malignancies, such as endemic Burkitt's lymphoma, nasopharyngeal carcinoma, certain B- and T-cell lymphomas, and approximately 50 percent of Hodgkin's disease cases (Ott et al., 1992; Weiss et al., 1989; zur Hausen et al., 1970). In 1969 a family was described with two brothers suffering from hypogammaglobulinemia and malignant lymphoma following infectious mononucleosis (Hambleton and Cottom, 1969). This was the first report of XLP-1 in the medical literature. In 1975, Purtilo et al. reported a large family, the Duncan kindred, in which multiple male members had died from fulminant infectious mononucleosis in early life, whereas others developed hypogammaglobulinemia after infectious mononucleosis or extranodal ileocecal malignant lymphoma later in life. The term "lymphoproliferative disease/syndrome" was coined to describe benign or malignant lymphoproliferations; in addition, the term "infiltrates composed of lymphocytes, plasma cells, and histiocytes, some containing erythrocytes" was used to describe the histological features of hemophagocytic lymphohistiocytosis (HLH) or virus-associated hemophagocytic syndrome (VAHS).

This X-chromosomally inherited syndrome with a high vulnerability to EBV infection was originally classified as Duncan disease (in reference to the first-described XLP-1 family). Subsequently, when more families with similar signs and symptoms had been identified, and it became clear that all those studied had a genetic defect localized at Xq25, the condition was named X-linked lymphoproliferative disease (Purtilo et al., 1991b). In 1978, an XLP-1 registry was established for the collection of clinical and laboratory data to facilitate basic research on the pathogenesis of XLP-1 and to develop new diagnostic and therapeutic tools for XLP-1 patients (Hamilton et al., 1980). As of the year 2000, 309 males with one or more XLP-1 phenotype(s) from 89 unrelated families had been registered (Seemayer et al., 1995; Sumegi et al., 2000). The disease has been shown to occur worldwide, with an estimated incidence of 1 to 3 per million males (Purtilo and Grierson, 1991). Primary EBV infection in

XLP-1 males may lead to severe and frequently fatal infectious mononucleosis, lymphoproliferative disorders usually of B-cell origin, dysgammaglobulinemia, and other less frequent manifestations (Seemayer et al., 1995). However, XLP-1 manifestations such as dysgammaglobulinemia or malignant lymphoma may develop in a similar frequency during the life of XLP-1 males who have never been exposed to EBV (Gross et al., 1994, 1995; Strahm et al., 2000; Sumegi et al., 2000), indicating that clinical manifestations of XLP-1 are not EBV-specific and that EBV may only act as a potent trigger of the earliest and most serious clinical manifestation of XLP-1 (i.e., fatal infectious mononucleosis).

In general, XLP-1 has an unfavorable prognosis. However, transplantation of hematopoietic stem cells from a suitable donor may cure this immunodeficiency.

The gene responsible for XLP-1, named *SH2D1A* (SH2 domain-containing gene 1A), was identified in 1998 independently by three groups (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). The gene encodes a small protein of 128 amino acids named SAP, for SLAM-associated protein, expressed in T lymphocytes and NK cells. SAP has the structure of a signaling adaptor containing a single SH2 domain. Through use of in vitro experiments and SAP-deficient mice, SAP has been shown to be involved in the signal transduction mediated by a family of transmembrane receptors, the signaling lymphocyte activation molecule (SLAM, also termed CD150 in humans) family.

CLINICAL PHENOTYPES AND PATHOLOGICAL MANIFESTATIONS

Prior to EBV infection, most boys carrying the defective XLP-1 gene are clinically healthy. Some of them exhibit subtle immunological abnormalities before EBV infection (see Laboratory Findings, below).

Exposure to EBV may result in three clinical phenotypes in males carrying the defective XLP-1 gene: fulminant and often fatal infectious mononucleosis occurs in more than half, lymphoproliferative disorders including malignant lymphoma develop in approximately one third of affected males, and dysgammaglobulinemia is found in one quarter of the patients (Table 44.1) (Seemayer et al., 1995; Sumegi et al., 2000). Less frequent manifestations are aplastic anemia, vasculitis, and lymphoid granulomatosis (each approximately 3 percent of cases) and bronchiectasis (Arico et al., 2001; Halasa et al., 2003; Schuster and Kreth, 2000; Seemayer et al., 1995; Strahm et al., 2000; Weeks et al., 2006). Several phenotypes (hypogammaglobulinemia, lymphoma, aplastic anemia) may manifest within the same XLP-1 patient over time. Moreover, considerable intra- and interfamilial variations in XLP-1 phenotypes exist.

XLP-1 has an unfavorable prognosis: by 10 years of age more than 70 percent of affected boys are dead; only 2 XLP-1 patients from the registered population of 272 males are older than 40 years of age (Seemayer et al., 1995). Only one man with XLP-1, who is not in the registry, is known to have survived for more than 60 years. He had a nonsense mutation in *SH2D1A* and a history of retropharyngeal lymphosarcoma at age 14 and intestinal lymphoma at 21 years; hypogammaglobulinemia was diagnosed at age 36 years; he died at age 64 from pancreatic cancer (H. D. Ochs, unpublished observation). Survival rates for fulminant infectious mononucleosis, lymphoproliferative disorders, dysgammaglobulinemia, and aplastic anemia are 4 percent, 35 percent, 55 percent, and 50 percent, respectively (Seemayer et al., 1995).

Table 44.1 **COMMON MANIFESTATIONS OF X-LINKED LYMPHOPROLIFERATIVE DISEASE TYPE 1 AND 2**

MANIFESTATIONS	XLP-1 (%)*	XLP-2 (%)*
HLH with EBV$^+$/FIM	45	49
HLH without EBV	7	24
Lymphoma	26	0
Hypo- or dysgammaglobulinemia	38	28
Splenomegaly without HLH	7	88
Hemorrhagic colitis	0	19

*Percentages calculated based on studies of Booth et al., 2011; Doshi et al., 2008; Marsh et al., 2010b; Pachlopnik Schmid et al., 2011; Rigaud et al., 2006; Sumegi et al., 2000; Worthey et al., 2011. Since XLP-1 and XLP-2 males may exhibit more than one phenotype, the sum of percentage values exceeds 100.

FIM, fatal infectious mononucleosis.

FULMINANT INFECTIOUS MONONUCLEOSIS

This is the most serious form of acute infectious mononucleosis, which has all the features of HLH. The median age of onset of illness is 2.5 to 3 years (range, 0.5–40 years) (Mroczek et al., 1987b; Sumegi et al., 2000); the median survival after onset of symptoms is 32 days (Mroczek et al., 1987b; Purtilo and Grierson, 1991). Signs and symptoms of disease are similar although more severe than those of infectious mononucleosis in older children and adults (Markin et al., 1987). Meningoencephalitis is common (Mroczek et al., 1987b; Schuster et al., 1993; Sullivan and Woda, 1989). During the course of fulminant mononucleosis, 89 percent of affected boys develop hepatic dysfunction, 81 percent have anemia, and 93 percent have thrombocytopenia (Mroczek et al., 1987b). Typically, polyclonal EBV-transformed B cells as well as CD4$^+$ and CD8$^+$ T cells (at approximately a 1:1 ratio of B to T cells) invade numerous organs, leading to infiltration and marked destruction of hematopoietic organs, liver, thymus, brain, heart, and other tissues (Markin et al., 1987; Mroczek et al., 1987a, 1987b; Sullivan and Woda, 1989). The extensive destruction of liver and bone marrow often leads to fulminant hepatitis and pancytopenia. In severe hepatitis, an intense periportal infiltration of EBV-positive B cells is typically found, surrounded by T cells, mainly of the CD8 phenotype (Markin et al., 1987). Liver failure with hepatic encephalopathy or hemorrhage involving the central nervous system, the gastrointestinal tract, or the lungs is the most frequent cause of death in these patients (Grierson and Purtilo, 1987).

HLH occurs in 90 percent of boys with fatal mononucleosis (Mroczek et al., 1987b; Seemayer et al., 1995) and is triggered

by EBV-infected cells. It is characterized by the appearance of numerous highly activated histiocytes and macrophages in different compartments (bone marrow, liver, lymph nodes). The development of HLH in bone marrow leading to pancytopenia has been shown to progress in three phases. Initially, moderate pancytopenia and a myeloid hyperplastic marrow develop, followed at 2 to 3 weeks by the appearance of numerous activated histiocytes and macrophages with erythrophagocytosis (Color Plate 44.I). Later, lymphoid infiltration of bone marrow occurs, often associated with necrosis and hemorrhage (Mroczek et al., 1987b). The pathogenesis of HLH in XLP-1 is thought to be linked to functional deficiency of activated NK and cytotoxic T cells that proliferate but do not eliminate EBV-infected cells see section "Function of the SAP Protein". The resulting overwhelming activation of histiocytes and macrophages is the direct result of uncontrolled release of tumor necrosis factor (TNF)-α and other cytokines ("cytokine storm") by activated T cells (Lay et al., 1997; Su et al., 1995).

HYPOGAMMAGLOBULINEMIA AND DYSGAMMAGLOBULINEMIA

This phenotype occurs in approximately 22 to 31 percent of affected males after EBV exposure. Median age at onset is 6 to 9 years (Grierson and Purtilo, 1987; Seemayer et al., 1995; Sumegi et al., 2000). Affected males exhibit varying degrees of hypogammaglobulinemia, and some may have increased serum IgM levels. Histologically, these patients present with extensive necrotic lesions in lymph nodes, thymus, bone marrow, and spleen. Long-term survivors have an inverted ratio of helper/cytotoxic T-cell subpopulations (CD4/CD8) in their peripheral blood (Grierson and Purtilo, 1987; Hügle et al., 2004; Malbran et al., 2004). Patients with hypogammaglobulinemia have a relatively favorable prognosis, particularly if they are treated with monthly intravenous immunoglobulin (IVIG) infusions.

XLP-1 patients with hypogammaglobulinemia resemble patients with common variable immunodeficiency (CVID), which is a heterogeneous disorder characterized by a decrease in all or some immunoglobulin isotypes and an impaired antibody response leading to increased susceptibility to infection (see Chapter 28). Several groups have reported mutations affecting the *SH2D1A* gene in patients with a CVID-like phenotype (Gilmour et al., 2000; Morra et al., 2001c; Nistala et al., 2001).

LYMPHOPROLIFERATIVE DISORDERS

Malignant lymphomas occur in approximately 30 percent of XLP-1 patients, often associated with dysgammaglobulinemia and/or fulminant mononucleosis. The median age of affected males at the time of diagnosis of lymphoma is 4 to 6 years (Grierson and Purtilo, 1987; Harrington et al., 1987; Seemayer et al., 1995; Sumegi et al., 2000). Presenting symptoms are fever, nausea, vomiting, lymphadenopathy, weight loss, and abdominal pain (Harrington et al., 1987).

Most lymphomas studied so far occurred at extranodal sites; in approximately 75 percent the ileocecal region was primarily affected (Harrington et al., 1987). Other common sites of these tumors are the central nervous system, liver, and kidneys. Over 90 percent of malignant lymphomas are of B-cell origin. These can be histologically subclassified as Burkitt's lymphomas (small noncleaved; 53 percent of B-cell lymphomas), immunoblastic lymphomas (18 percent), large noncleaved lymphomas (12 percent), small cleaved or mixed-cell lymphomas (12 percent), and unclassifiable lymphomas (5 percent) (Harrington et al., 1987). Only five cases have been classified as T-cell lymphomas (6 percent of all lymphomas in XLP-1 patients studied to date). Three brothers of one XLP-1 family developed Hodgkin's disease following EBV infection (approximately 3 percent of all lymphomas in XLP-1 patients studied to date), a fourth brother died from malignant non-Hodgkin's lymphoma (Donhuijsen-Ant et al., 1988). Malignant lymphomas with different histological subclassification may occur within the same family (Donhuijsen-Ant et al., 1988; Schuster et al., 1994). In at least four cases of Burkitt's lymphomas, the characteristic 8;14 translocation (Egeler et al., 1992; Strahm et al., 2000; Williams et al., 1993) and in one case the 8;22 translocation (Turner et al., 1992) were demonstrated.

The prevalence of malignant lymphomas in XLP-1 males is higher than in patients with other inherited immunodeficiencies such as the Wiskott-Aldrich syndrome (Chapter 43), and the risk of developing a lymphoma has been estimated to be approximately 200 times greater than that in the general population (Grierson and Purtilo, 1987). In the case of Burkitt's lymphoma, by using a mathematical model simulating lymphomagenesis, the cumulative lymphoma prevalence has been estimated to be 0.002 percent by the chronological age of 18 years in the general population, 0.15 percent by age 6 years for endemic Burkitt's lymphoma, 3 percent by age 10 years for Burkitt's lymphoma in children with AIDS, and 25 percent by age 7 years for Burkitt's lymphoma in boys with XLP-1 (Ellwein and Purtilo, 1992). This markedly increased risk in XLP-1 males, theoretically predicted and later statistically confirmed, may indirectly reflect the enhanced proliferation of EBV-infected B cells, which may be due to impaired immunosurveillance by T lymphocytes and NK cells.

Characteristics that may distinguish malignant lymphomas in XLP-1 patients from other malignant lymphomas include a family history of XLP-1, the early age of clinical manifestation (infectious mononucleosis), the ileocecal localization of the majority of XLP-1–associated lymphomas, and additional XLP-1 phenotypes in the same affected male (such as dysgammaglobulinemia and severe or fulminant infectious mononucleosis). Approximately 30 to 35 percent of XLP-1 patients with malignant lymphoma survive for longer periods (>10 years) (Purtilo and Grierson, 1991; Seemayer et al., 1995).

OTHER RARE XLP-1 PHENOTYPES

A small number of XLP-1 patients (approximately 3 percent of cases) develop isolated bone marrow aplasia (either pancytopenia or pure red cell aplasia) in the absence of any evidence for HLH. A similar percentage of affected patients may develop

necrotizing lymphoid vasculitis or lymphoid granulomatosis of the lung or the central nervous system leading to extensive tissue damage (Loeffel et al., 1985; Dutz et al., 2001; Kanegane et al., 2005; Seemayer et al., 1995). Another rare clinical manifestation of XLP-1 is bronchiectasis, which has been found in association with (Dutz et al., 2001; Hügle et al., 2004; Mehta al., 1999) and without (Strahm et al., 2000) dysgammaglobulinemia. In certain cases, HLH may be the only clinical manifestation in males with XLP-1 (Arico et al., 2001). Recently, a *Helicobacter pylori*-negative, EBV-negative chronic active atrophic pangastritis, with superficial ulcer formation, foveolar hyperplasia, glandular dilatation, and ultimately pseudopyloric intestinal metaplasia was found as a rare clinical phenotype in monozygotic twins with XLP-1 (Rougemont et al., 2008). One XLP-1 patient developed EBV encephalitis, central nervous system lymphoproliferative disease, and lymphoma, as well as diffuse fusiform aneurysmal dilation of intracranial vessels (Weeks et al., 2006). A young adult lacking the typical symptoms of XLP-1 was recently reported to have developed generalized skin manifestations resembling scleroderma with scarring of the subcutaneous tissue at 8 years of age. As the skin pathology progressed, he showed movement limitation of elbows and knees and developed severe mutilation of hands and feet (Mejstríková et al., 2012). Interestingly, a maternal cousin with the same deletion of the entire SH2D1A gene presented as a teenager with a classic XLP-1 phenotype.

CLINICAL MANIFESTATIONS AND IMMUNOLOGICAL FINDINGS IN XLP-1 MALES WITH NO EVIDENCE OF PREVIOUS EBV EXPOSURE

Thirty-eight (symptomatic) males with XLP-1, representing 12.5 percent of 304 registered males with clinical manifestations of XLP-1, had no evidence of prior EBV exposure (Sumegi et al., 2000). The number and percentage may be even higher, since the exact number of asymptomatic XLP-1 males is not known.

Interestingly, EBV-negative XLP-1 patients developed dysgammaglobulinemia and/or malignant lymphoma in a similar percentage to that of the EBV-positive group (Sumegi et al., 2000). In the EBV-negative group, median age at the onset of dysgammaglobulinemia was 4.5 years; for lymphoproliferative disease it was 8 years, which is not statistically different from the EBV-positive group. Infectious mononucleosis, which may be also caused by other herpesviruses such as cytomegalovirus (CMV), human herpes virus type 6 (HHV-6), and HHV-7 or by *Toxoplasma,* was never observed in the EBV-negative group. Survival for the EBV-negative group as a whole was significantly better, but this result is entirely accounted for by the poor survival of fulminant infectious mononucleosis in the EBV-positive group (Sumegi et al., 2000).

Thirty-two EBV-negative males carrying the defective XLP-1 gene as shown by restriction fragment length polymorphism (RFLP) analysis—but not by sequencing—have been further immunologically investigated (Grierson et al., 1991; Gross et al., 1994; Seemayer et al., 1995). Among these, only five males were clinically healthy and had normal serum immunoglobulin levels. The remaining 27 males showed, prior to EBV exposure, one or both of the following XLP-1 phenotypes: 17 subjects had elevated serum IgA or IgM and/or variable deficiency of IgG, IgG1, or IgG3, and 18 males developed lymphoproliferative disease (15 of B-cell origin, three of T-cell origin) without serological and/or genomic evidence of previous EBV infection (22 percent of all lymphomas in XLP-1 studied thus far). Eight of these 27 males (30 percent) had both dysgammaglobulinemia and lymphoma. These phenotypes resemble that of late-onset CVID. The findings strongly suggest that the immunodeficiency in XLP-1 is not strictly EBV-related.

In the few XLP-1 males without evidence of prior EBV infection studied thus far, the number of peripheral T, B, and NK cells; the expression of CD3, CD4, CD8, and CD28; the CD4/CD8 ratio; the proliferative response to phytohemagglutinin (PHA), con-canavalin A (ConA), and pokeweed mitogen (PWM); and NK-cell activity seem to be normal (Gilleece et al., 2000; Strahm et al., 2000; Sullivan and Woda, 1989). In one XLP-1 patient interferon-γ (IFN-γ) secretion by peripheral blood mononuclear cells was shown to be increased (ELISPOT assay) following stimulation with phorbol myristate acetate (PMA) but was decreased after stimulation with anti-CD3 and/or anti-2B4 (Sharifi et al., 2004).

The pattern of dysgammaglobulinemia appears to be due to a partial failure of the patient's B cells to undergo isotype switching from IgM to IgG. Moreover, EBV-seronegative (as well as EBV-seropositive) XLP-1 patients exhibit a deficient switch to IgG antibody production following secondary challenge with bacteriophage ΦX174, a finding that has been used in the past for diagnostic evaluation of males at risk for XLP-1 (Purtilo et al., 1989). However, for various reasons this test possesses only limited diagnostic validity and reliability, and may lead to false-positive and false-negative results (Purtilo, 1991). Therefore, the diagnosis of XLP-1 in EBV-negative asymptomatic males depends on direct mutation analysis of the *SH2D1A* gene. The finding of abnormal immunoglobulin and IgG subclass concentrations (vide supra) in the serum of males at risk for XLP-1 is suggestive but not diagnostic. Because of the high prevalence of EBV in the general population, most males with XLP-1 will be infected with EBV in childhood. About 58 percent of them will develop the most severe XLP-1 phenotype (i.e., fatal infectious mononucleosis).

LABORATORY FINDINGS IN XLP-1 MALES DURING ACUTE EBV INFECTION

During fulminant infectious mononucleosis, a triphasic process develops along a continuum for several weeks within the blood and bone marrow (Purtilo, 1991). Initially (for 1–2 weeks), the leukocyte count is elevated, mostly because of increased numbers of atypical lymphoid cells, most of which are activated T cells. This abnormality is similar to but more extensive than that of immunocompetent children with uncomplicated infectious mononucleosis. At this time the bone marrow is hyperplastic with granulocytic hyperplasia with a left shift of maturation (Mroczek et al., 1987b). Later, severe pancytopenia develops (median leukocyte count,

1.5×10^9/L; median platelet count, 35.5×10^9/L; median hemoglobin level, 8.5 g/dL). The bone marrow shows extensive infiltration by lymphoid cells consisting of activated T cells, immunoblasts, and plasma cells. This is associated with cellular necrosis and HLH (Color Plate 44.I) (Mroczek et al., 1987b). Terminally, the marrow shows massive necrosis with severe cellular depletion and marked histiocytic hemophagocytosis (Purtilo, 1991).

Elevation of serum transaminases, lactic dehydrogenase, and bilirubin, reflecting hepatic involvement, is found in all affected boys with fulminant mononucleosis. Hepatic encephalopathy or severe hemorrhages (central nervous system, gastrointestinal tract, lungs) due to liver failure are the most frequent cause of death in XLP-1 boys with fatal mononucleosis (Grierson and Purtilo, 1987).

The age range of XLP-1 males with fulminant mononucleosis is 5 months to 17 to 40 years (Mroczek et al., 1987b; Sumegi et al., 2000); the median age is 2.5 to 3 years. In most cases there is direct evidence of EBV infection as shown by the presence of heterophile antibodies and/or positive EBV-specific serology and/or the presence of EBV antigens or DNA in lymphoid tissues (positive in 100 percent of cases) (Falk et al., 1990; Mroczek et al., 1987b). Demonstration of EBV genomes in serum by polymerase chain reaction (PCR) is a readily available test to indicate EBV infection (Sumazaki et al., 2001).

IMMUNOLOGICAL STUDIES OF XLP-1 PATIENTS AFTER EBV INFECTION

Subjects with XLP-1 surviving EBV infection exhibit, as a rule, combined T- and B-cell defects. Most XLP-1 patients have normal numbers of peripheral blood B and T cells. The proliferative responses of lymphocytes to B- and T-cell mitogens such as PHA, ConA, and PWM and to anti-IgM antibody were shown to be abnormally low during and after primary EBV infection in four of four XLP-1 patients (Sullivan and Woda, 1989). In contrast, others have found normal lymphocyte proliferation in response to PHA, PWM, EBV, and *Staphylococcus aureus* strain Cowan (SAC) in 9/9 XLP-1 patients (Arkwright et al., 1998; Lindsten et al., 1982). A decreased ratio of $CD4^+$ to $CD8^+$ T cells with a predominance of $CD8^+$ cells has been observed in most XLP-1 patients (Hügle et al., 2004; Lindsten et al., 1982). The in vitro production of immunoglobulins by peripheral blood lymphocytes in response to PWM, EBV, and SAC was markedly decreased compared with healthy controls (Lindsten et al., 1982). Furthermore, in XLP-1 patients with hypogammaglobulinemia, the in vitro synthesis of IgG, IgM, and IgA by B lymphoblastoid cell lines in the presence of autologous T cells was markedly decreased (Lai et al., 1987; Yasud et al., 1991). The degree of this T-cell–mediated "suppression" of Ig synthesis correlated with the decreased serum levels of IgM and IgG in these patients (Yasuda et al., 1991).

XLP-1 patients have a marked reduction in the number of circulating $CD27^+$ memory B cells, which undergo normal somatic hypermutations, but a complete absence of switched memory B cells (Ma et al., 2005, 2006; Malbran et al., 2004). $CD4^+$ cells from XLP-1 patients do not efficiently differentiate in vitro into IL-10–secreting T-helper cells and show reduced expression of inducible costimulator (ICOS) (Ma et al., 2005). These data suggest that in XLP-1 the B-cell defects are mainly extrinsic. More recently, it was reported that XLP-1 patients have increased numbers of functionally immature "transitional" CD24highCD38high B cells, which may further contribute to the humoral immunodeficient state (Cuss et al., 2006).

T cells from XLP-1 patients failed to secrete normal amounts of IFN-γ following stimulation with autologous B lymphoblastoid cell lines or via the SLAM family receptor 2B4 (Sharifi et al., 2004; Yasud et al., 1991). In contrast, in three unrelated XLP-1 patients the frequency of IFN-γ–producing $CD3^+$ T cells (measured intracytoplasmatically by FACS analysis after stimulation of the cells with PMA and ionomycin) was markedly increased (Ehl et al., 2002; Hügle et al., 2004). Furthermore, mononuclear cells from other XLP-1 patients spontaneously produced normal or elevated levels of IFN-γ during EBV infection (Okano et al., 1990; Sullivan, 1983). It is therefore not clear if deficient IFN-γ production in XLP-1 is a primary defect or only secondary to EBV infection. Skin tests with various antigens (purified protein derivative [PPD], tetanus toxoid, *Candida*, and mumps) are frequently negative in XLP-1 patients (Donhuijsen-Ant et al., 1988; Sullivan, 1983). A direct role of SAP in early cell antigen receptor-mediated signaling via CD3 was suggested by defective upregulation of IL-2 and IFN-γ production by herpesvirus saimiri-immortalized $CD4^+$ T cell (Nakamura et al., 2001) and by defective IL-2 production, CD25 expression, and homotypic cell aggregation (Sanzone et al., 2003).

NK cell activity of XLP-1 males was found to be normal before, high at the time of, and low after EBV primary infection, results suggesting that this defect might be acquired during EBV infection (Argov et al., 1986; Okano et al., 1990; Rousset et al., 1986; Sullivan et al., 1980, 1983; Sullivan and Woda, 1989). Recent studies, however, have shown that in XLP-1 patients NK-cell cytotoxicity mediated by the NK-cell activating receptor 2B4 seems to be selectively impaired, whereas NK-cell activation mediated by NKp46, NKp44, NKp30, CD2, or CD16 as well as natural cytotoxicity against K562 cells and major histocompatibility complex (MHC) class I-deficient cells seems not be affected (Benoit et al., 2000; Nakajima et al., 2000; Parolini et al., 2000; Tangye et al., 2000b).

Patients with XLP-1 have a nearly complete absence of CD1d-restricted NKT lymphocytes (Chung et al., 2005; Nichols et al., 2005; Pasquier et al., 2005), which are important for bridging innate and adoptive responses (Bendelac et al., 2007; Latour et al., 2007). Studies in mice showed that in the absence of SAP, the development of NKT cell is blocked early in the thymus at the positive selection step (Griewank et al., 2007; Pasquier et al., 2005). The exact role of NKT cells in the control of EBV infection is not known, but interestingly, a subset of $CD8\alpha\alpha$ NKT cells with cytolytic functions was shown to suppress the proliferation and the expansion of activated $CD8^+$ T cells in vitro. Importantly, expansion of anti-EBV $CD8^+$ T cells was reduced in these conditions (Ho et al., 2004).

EBV-specific T-cell immune function has been studied in only a few XLP-1 patients. Three of eight XLP-1 patients were shown to exhibit EBV-specific memory T-cell activity as measured by an outgrowth inhibition assay (regression assay)

(Harada et al., 1982). In two XLP-1 patients with hypogammaglobulinemia, EBV-specific, HLA-restricted cytotoxicity was demonstrated (Rousset et al., 1986). In recent studies, EBV-specific $CD8^+$ T cells from XLP-1 patients exhibited markedly decreased cytotoxic activity against autologous B-cell lines (Dupre et al., 2005; Hislop et al., 2010; Sharifi et al., 2004). The deficient EBV-specific cytotoxicity could be fully reconstituted by retroviral gene transfer of the *SH2D1A* gene into the T-cell lines. These findings suggest that in XLP-1 patients the lack of SAP may result in severe disruption of cytotoxic T-lymphocyte (CTL) function. XLP-1 patients have normal numbers of EBV-specific $CD8^+$ T cells that are extremely differentiated as defined by loss of CCR7 and CD27, low telomerase activity, and very short telomeres (Plunkett et al., 2005). These observations suggest that excessive proliferation of $CD8^+$ T cells may lead to end-stage differentiation and loss of EBV-specific $CD8^+$ T cells through replicative senescence. Lack of cytotoxic T cells may contribute to the development of EBV-associated B-cell lymphomas.

Interestingly, some of these pathological findings in XLP-1 patients can also be found transitorily and for a short period in patients with uncomplicated acute infectious mononucleosis. T lymphocytes obtained during acute infectious mononucleosis proliferate poorly in response to various mitogens such as PHA, ConA, and PWM as well as to antigens such as tetanus toxoid and *Candida albicans* (Tosato, 1989). Moreover, patients with acute infectious mononucleosis show low or absent delayed-type hypersensitivity reactions when challenged with recall antigens (Tosato, 1989) and respond poorly to the T-cell–dependent neoantigen bacteriophage φX174, both in vivo and in vitro (Junker et al., 1986).

T-cell activation during acute infectious mononucleosis leads to the amplification of $CD8^+$ cytotoxic T cells, of which only a fraction is EBV-specific and HLA-restricted (Callan et al., 1996; Tomkinson et al., 1989). It also leads to the proliferation of $CD8^+$ T cells, which seem to be able to inhibit proliferation and immunoglobulin production by B cells as well as to inhibit nonactivated T cells in a probably virus-nonspecific, HLA-nonrestricted manner (Tosato, 1989; Tosato et al., 1982; Wang et al., 1987). In healthy immunocompetent individuals, both cytotoxic and suppressor functions, displayed by $CD8^+$ T cells during infectious mononucleosis, appear to modulate and finally self-limit the course and severity of EBV infection. As a consequence, aberrant self-destructive killer cell populations and/or suppressor cells are not downregulated or eliminated and may therefore proliferate in an uncontrolled manner, leading to tissue and organ destruction (as found in fatal mononucleosis) and later on to hypogammaglobulinemia and diminished immunosurveillance against EBV-infected B cells, with an enhanced risk of B-cell lymphomas.

EBV–HOST INTERACTION IN X-LINKED LYMPHOPROLIFERATIVE DISEASE

Males with XLP-1 show a range of abnormal antibody responses after primary EBV infection. Antibody titers to the EBV nuclear antigen (EBNA) are decreased or absent, which may indirectly reflect a T-cell immunodeficiency, as this is also seen in other inherited or acquired immunodeficiencies (Okano et al., 1992; Purtilo et al., 1985). Antibodies against the EBV capsid antigen (VCA) are variable in XLP-1 males. In rare instances, XLP-1 patients may not be able to produce any EBV-specific antibodies (anti-VCA-IgM, anti-VCA-IgA, anti-VCA-IgG, or anti-EBNA) despite overwhelming infection with EBV (Dutz et al., 2001; Hayoz et al., 1988; Turner et al., 1992). In these cases the detection of EBV genomes by PCR or the demonstration of EBNA by immunohistochemical staining in lymphoid tissue will document EBV infection in spite of seronegativity. In a large study of patients with sporadic or XLP-1–associated fatal mononucleosis, it was shown that 13 of 15 patients had histological changes that were characteristic for polyclonal proliferation of B cells that expressed, in most cases, all EBV nuclear antigens (EBNA1–6), an expression pattern seen also in lymphoblastoid B-cell lines from healthy controls. In two cases, the B cells were of monoclonal origin and expressed only EBNA1, a pattern also found in Burkitt's lymphoma (Falk et al., 1990).

In one patient with a nonsense mutation in *SH2D1A* (Hügle et al., 2004), EBV DNA and proteins could be detected in most of his peripheral T cells during the acute phase of infectious mononucleosis (Baumgarten et al., 1994). In general, B cells are the primary target of EBV in patients with infectious mononucleosis and other EBV-associated disorders (Kurth et al., 2000). EBV-infected T cells have been found in only one case of fatal acute mononucleosis (Mori et al., 1992) and in patients with severe chronic active EBV (CAEBV) infection (Ohga et al., 2001; Quintanilla-Martinez et al., 2000). Whether XLP-1 is associated with an increased rate of EBV infection of T cells is unknown, but this seems unlikely, since only 6 percent of XLP-1–associated lymphomas are of T-cell origin (90 percent B-cell lymphoma, 4 percent Hodgkin's disease) (Schuster and Kreth, 2000; Seemayer et al., 1995).

Normally, EBV type 1 is found predominantly in immunocompetent subjects after EBV infection. In immunodeficient individuals (HIV infection, organ transplantation), increased infection rates with EBV type 2 have been observed (Sculley et al., 1990). EBV strains isolated from XLP-1 patients have not yet been genotyped systematically. In at least two unrelated XLP-1 patients, EBV type 2 could be identified in lymphoid tissues (Mulley et al., 1992; Vowels et al., 1993). In five XLP-1 patients studied so far, EBV type 1 was found in lymphoma biopsy or peripheral blood (Chaganti et al., 2008; Schuster et al., 1996). It was further shown that in XLP-1 patients the circulating EBV load is concentrated within a small population of nonswitched $CD27^+$ B cells that were IgD^+IgM^+ and lacked IgG or IgA expression (Chaganti et al., 2008).

Patients with XLP-1 are vulnerable only to EBV, whereas the immune response to other herpesviruses, such as CMV, herpes simplex virus (HSV), HHV-6, HHV-7, and HHV-8, does not seem to be impaired.

MOLECULAR BASIS

In 1987 the XLP-1 gene locus was mapped to the long arm of the X chromosome at Xq25 through RFLP analysis with polymorphic DNA markers from Xq24 to Xq26 (Skare et al.,

1987, 1989a, 1989b, 1989c; Sylla et al., 1989). In several families with XLP-1, different partly overlapping deletions in the Xq25 region were identified and further characterized (Coffey et al., 1998, Lamartine et al., 1996; Lanyi et al., 1997; Porta et al., 1997; Sanger et al., 1990; Skare et al., 1993; Wu et al., 1993; Wyandt et al., 1989). In 1998, three groups independently identified the gene defective in XLP-1 by positional cloning (Coffey et al., 1998; Nichols et al., 1998) or through a functional approach (Sayos et al., 1998). The gene (and its encoded protein) was originally designated *SH2D1A* (Coffey et al., 1998), *SAP* (Sayos et al., 1998), or *DSHP* (Nichols et al., 1998). The gene name approved by the Human Gene Nomenclature Committee (HGNC) is *SH2D1A.* The protein encoded by *SH2D1A* is designated as SAP (for SLAM-associated protein). The human, monkey, and mouse *SH2D1A* genes consist of four exons and three introns spanning 25 kb (Coffey et al., 1998; Morra et al., 2001a, 2001d; Wu et al., 2000). The human *SH2D1A* gene encodes a small cytoplasmic protein of 128 amino acid residues consisting of a single SH2 domain, and a 25-amino acid carboxy-terminal tail (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998) (Fig. 44.1a). Two *SH2D1A* mRNA species with a size of 2.5 kb and 0.9 kb are transcribed (Sayos et al., 1998).

The highest level of human *SH2D1A* mRNA expression has been found in the thymus and to a lesser extent in spleen, liver, lymph nodes, and other lymphoid organs, as well as in peripheral blood lymphocytes (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). SAP is expressed in all major subsets of human T cells, with activated cells expressing the highest amounts, as well as in $CD56^+CD3^+$ NK cells (Coffey et al., 1998; Nagy et al., 2000, 2002; Nichols et al., 1998; Sayos et al., 1998). *SH2D1A* mRNA and SAP expression has been reported to occur in different B- and T-cell lymphomas and Hodgkin's disease as well as in different T-cell lines, but not in EBV-immortalized B lymphoblastoid cell lines (Kis et al., 2003; Nagy et al., 2000, 2002; Nichols et al., 1998; Sayos et al., 1998). Similarly, in mice SAP expression is predominantly found in T cells and NK cells (Sayos et al., 2000; Wu et al., 2000). In mice and in humans, SAP expression is downregulated in $CD4^+$ and $CD8^+$ T cells upon anti-CD3 stimulation (Wu et al., 2000; D. Howie, unpublished data).

SAP is not unique and shares the same overall gene and protein organization with related molecules named Ewing's sarcoma-activated transcript 2 (EAT-2) and EAT-2-related transducer (ERT; only found in rodent; in humans *ERT* is a pseudogene). This family of molecules is designated as the SAP family of adaptors. EAT-2 is found in NK cells, dendritic cells (DCs), and macrophages, whereas ERT is present only in NK cells (Roncagalli et al., 2005).

FUNCTION OF THE SAP PROTEIN

Based on the single SH2 domain composition (Fig. 44.1a), it was postulated that SAP might be involved in protein phosphorylation-mediated signal transduction events (Fig. 44.2). The first clue supporting this notion was provided by the finding that SAP interacts by the way of its SH2 domain with a tyrosine-based motif in the cytoplasmic domain of the transmembrane receptor SLAM (Sayos et al., 1998). Further experiments, including random peptide library screenings, showed

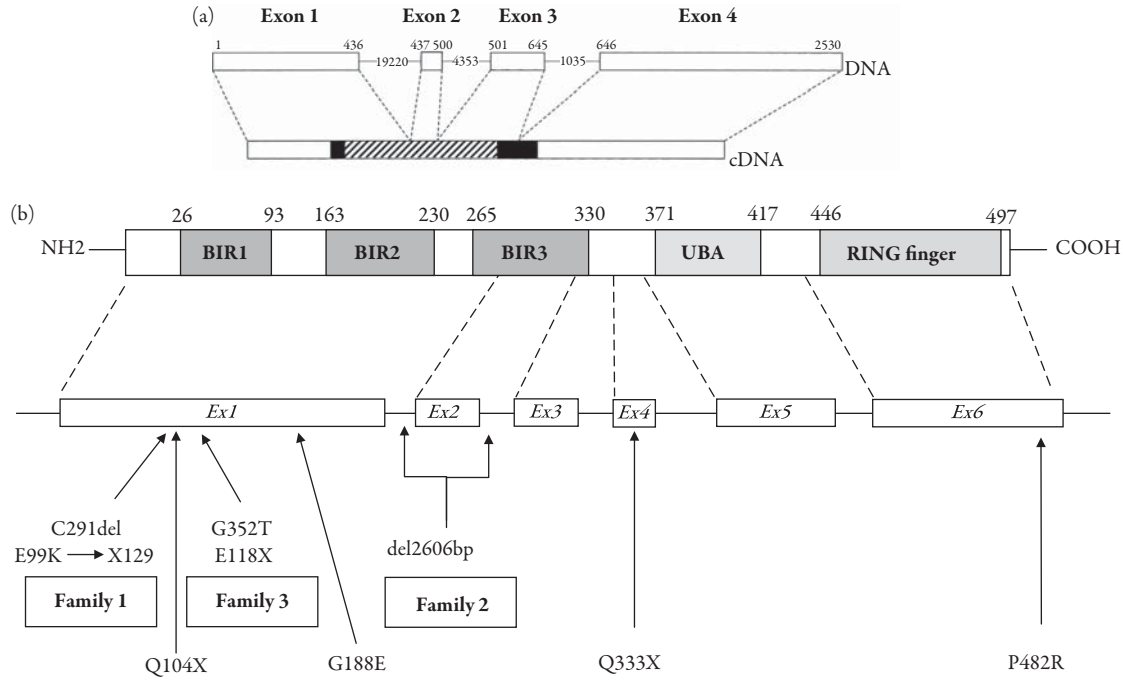

Figure 44.1 Organization of the *SH2D1A* gene (a) and the *XIAP* gene (b). *SH2D1A* gene: black regions: coding sequences; portion with diagonal pattern: SH2 domain (adapted from Coffey et al., 1998). *XIAP* gene: light gray boxes: BIR (baculovirus IAP repeats) domains; dark gray box: ubiqutin binding domain (UBA); RING finger domain or E3 ubiquitin ligase (gray box). Mutations found in XLP-2 patients are indicated in the exon structure of the gene. Three mutations (G188E; deletion Exon 2; P482R) are known to be associated with decreased or normal expression of XIAP. Deletion of Exon 1 to 5 is not shown.

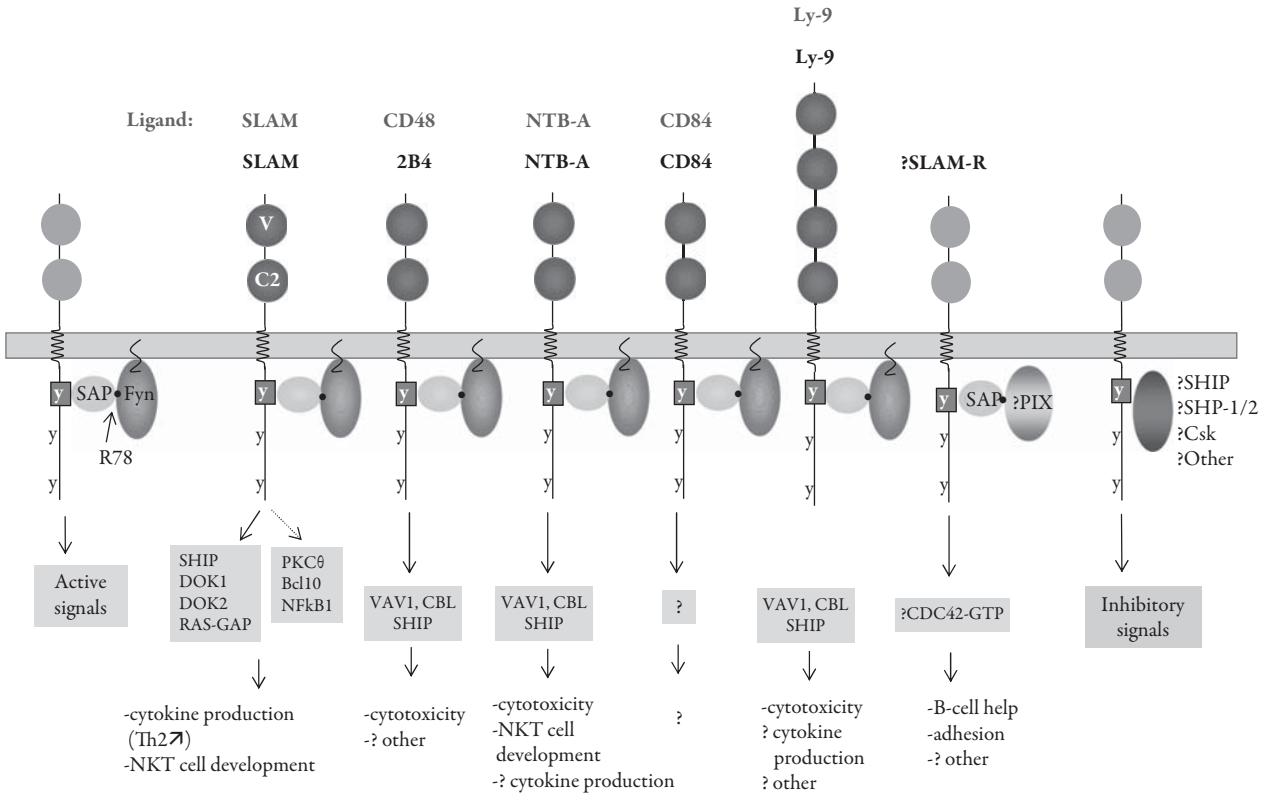

Figure 44.2 A model of regulation of SLAM receptors by SAP (adapted from Schwartzberg et al., 2009, and Veillette et al., 2009). The adaptor molecule SAP is required for mediating active biochemical signals from SLAM family receptors (SLAM-R) by its capacity to recruit and activate the protein tyrosine kinase (PTK) Fyn via its R78 residue that binds to the Fyn SH3 domain. The SLAM receptor family comprises six members: SLAM, 2B4, NTB-A, CD84, Ly-9, and CRACC. With the exception of CRACC, all members contain in their intracytoplasmic domain one or several tyrosine-based motifs that recruit SAP when phosphorylated. All SLAM receptors are self-ligands, with the exception of 2B4, which recognizes CD48. Engagement of SLAM receptors in T and NK cells results in the formation of a transducing module made of SLAM-R, SAP, and Fyn, which promote immune-cell functions such as cytokine production, cytotoxicity, and NKT-cell development.

The T-help to B-cell function (B-cell help), which depends on an adhesion process, is dependent on SAP, but the SLAM receptors that contribute to this function are not known. Moreover, it is not dependent on the PTK Fyn. In this context, it is possible that SAP recruits the exchange factor PIX, which has been shown to bind to SAP via the R78 residue like Fyn. In the absence of SAP, SLAM-R–mediated active signals are shifted toward inhibitory signals. SAP is also a "blocker" molecule that interferes with the intrinsic capacity of SLAM receptors to deliver inhibitory signals by regulating and/or displacing the recruitment of inhibitory molecules such as the 5′ inositol phosphatase (SHIP).

that the SAP and EAT-2 SH2 domains bind with high affinity and specificity to the sequence TIpYxxV/I (where T is threonine, I is isoleucine, Y is tyrosine, and V is valine) (Hwang et al., 2002; Poy et al., 1999). One or more copies are found in the cytoplasmic domain of all SLAM-related receptors, with the exception of CRACC. SLAM-related receptors form the SLAM family, which includes SLAM, 2B4, CD84, NTB-A (Ly-108 in mouse), Ly-9, and CRACC. All members of the SLAM family share a common structural organization, which consists of an extracellular domain with two or four immunoglobulin (Ig)-like domains, a single transmembrane portion, and an intracytoplasmic tail with tyrosine-based motifs. By way of their extracellular domain, SLAM family receptors interact with their ligands via homotypic interactions, with the exception of 2B4, which interacts with the CD48 molecule. These interactions are thought to participate in adhesion processes during cell–cell contact and to trigger intracellular signaling (Cao et al., 2006; Yan et al., 2007).

Because SAP is composed almost entirely of an SH2 domain, it was proposed at first that SAP might function as a natural competitor or blocker of SH2 domain-mediated interactions. The "blocking model" was supported by observations that SAP could prevent binding of SLAM and 2B4 to the SH2 domain-containing protein tyrosine phosphatase SHP-2 in nonimmune cells (Sayos et al., 1998; Tangye et al., 1999). However, the capacity of SLAM receptors to bind SHP-2 in the absence of SAP was not confirmed in several systems including immune cells derived from XLP-1 patients. (Latour et al., 2001; Nakajima et al., 2000; Parolini et al., 2000). Nonetheless, SAP might prevent the binding of SLAM-related receptors with other types of SH2-containing molecules.

An other important clue regarding the mechanism of action of SAP was given by the findings that SAP is absolutely required for SLAM-triggered protein tyrosine phosphorylation in T cells, due to the capacity of SAP to recruit and activate the Src-related protein tyrosine kinase (PTK) FynT (Latour et al., 2001). The ability of SAP to recruit FynT involves a direct interaction between the SAP SH2 domain and the FynT SH3 domain through a second binding surface centered on arginine 78 in the SAP SH2 domain (Chan et al., 2003;

Latour et al., 2003). The SAP–FynT interaction is very specific given that the arginine 78-based motif of SAP does not seem to bind other members of the Src family. Nevertheless, this motif can also associate with another SH3 domain-containing molecule, PAK-interacting exchange factor (PIX) (Gu et al., 2006). Therefore, SAP appears to behave as a true adaptor protein that couples SLAM family receptors to active biochemical signals. Contrary to SAP, EAT-2 lacks the arginine 78-based motif and is unable to bind to the FynT SH3 domain (Latour and Veillette, 2003). In agreement, recent reports indicate that the mechanism of EAT-2 action is distinct from those of SAP (Dong et al., 2009; Roncagalli et al., 2005).

Recent progress indicates that SLAM family receptors play multiple roles in immune responses and that in the absence of SAP dysfunctions of SLAM receptors account for the pathophysiology of XLP-1 (Dong et al., 2009; Rezaei et al., 2011). In T cells, in the presence of SAP, engagement of SLAM triggers a rapid tyrosine phosphorylation signal that results in the recruitment and the activation of FynT by SAP (Latour et al., 2001). This mechanism allows tyrosine phosphorylation of SLAM itself and, as a result, the recruitment of the 5′ inositol phosphatase SHIP-1 via binding of the SHIP SH2 domain to SLAM. This binding induces tyrosine phosphorylation of SHIP-1 and subsequent recruitment of the adaptor molecules Dok1, Dok2, and Shc as well as of the Ras-GTPase-activating protein (Ras-GAP). In murine T cells, the SLAM-SAP-Fyn signaling module selectively inhibits IFN-γ production and enables IL-4 production, hence promoting Th2 responses. In SAP-deficient T cells, IFN-γ production is increased compared with that of normal cells (Howie et al., 2002a; Wu et al., 2001). By contrast, IL-4 production by $CD4^+$ T cells was compromised in SAP- or Fyn-deficient mice as well as in mice expressing the SAP R78A mutant that fails to associate with Fyn (Cannons et al., 2004; Davidson et al., 2004; Wu et al., 2001). In this context, the signaling cascade triggered by the interaction of SLAM with SAP appears also to involve the protein kinaseCθ (PKCθ) and its downstream effectors Bcl-10 and NFκB1 (Cannons et al., 2004).

In NK cells expressing SAP, 2B4 engagement results in a specific protein tyrosine phosphorylation signal that involves 2B4, PLC-γ1, LAT, the exchange factor Vav-1, the ubiquitin ligase c-Cbl, and to a lesser extent SHIP-1 (Bottino et al., 2000; Chen et al., 2004; Watzl et al., 2000). Similar to SLAM signaling, 2B4-mediated protein phosphorylation is strictly dependent on SAP, and on the ability of SAP to recruit FynT. Likewise, the ability of 2B4 engagement to stimulate NK cell-mediated killing requires SAP and Fyn expression, as revealed by the observations that 2B4-mediated cytotoxicity is severely compromised in NK but also in $CD8^+$ T cells derived from XLP-1 patients and in Fyn-deficient mice (Benoit et al., 2000; Bloch-Queyrat et al., 2000; Dupré et al., 2005; Hislop et al., 2010; Nakajima et al., 2000; Parolini et al., 2000; Sharifi et al., 2004; Tangye et al., 2000b).

In the same way, the capacity of NTB-A (Ly108 in mouse) and Ly-9 to transduce active signals also depends on SAP and its aptitude to recruit FynT (Simarro et al., 2004; Zhong et al., 2008). In mouse T cells, stimulation of NTB-A results in the formation of the NTB-A-SAP-FynT signaling module that promotes a specific protein tyrosine phosphorylation signal similar to that triggered by 2B4 (Zhong at al., 2008). In agreement with these findings, NTB-A efficiently promotes cytotoxicity in human NK cells in a SAP-dependent manner as NTB-A–mediated cytotoxicity is impaired in XLP-1 patients similarly to 2B4 (Bottino et al., 2001). When engaged Ly-9 also forms a signaling module with SAP and FynT that mediates a tyrosine phosphorylation signal involving SHIP, Dok2, and the association with the adaptor Grb2 (Martin et al., 2005; Sylvain Latour and Francis Relouzat, unpublished data).

In NKT cells, a signaling module involving at least two SLAM receptors, SLAM and NTB-A, is thought to be important for NFκB activation, which is required for NKT-cell development (Bendelac et al., 2007; Griewank et al., 2007).

Therefore, the capacity of SAP to recruit FynT enables SLAM family receptors to mediate protein tyrosine phosphorylation signals. These signals appear to be essential for the function of SAP in immune cells, given that most of the immune defects caused by the SAP deficiency in T cells, NK cells, and NKT cells are also found in mice lacking Fyn. However, recent findings indicated that some of the functions of SAP may occur through a FynT-independent mechanism affecting T cells leading to humoral immune defects (Cannons et al., 2006; McCausland et al., 2007).

Importantly, early studies of human NK cells from XLP-1 patients revealed that in the absence of SAP, the activating function of 2B4 and NTB-A is shifted toward inhibition of NK-cell–mediated cytotoxicity (Bottino et al., 2001; Parolini et al., 2000) (Fig. 44.3). Similar observations have been reported with $CD8^+$ T cells from SAP-deficient patients. While EBV-transformed lymphoid blast cell lines that did not express SLAM-R were efficiently recognized and killed by autologous SAP-deficient $CD8^+$ T-cell clones, LCLs expressing CD48 and NTB-A were not killed. Importantly, their recognition and their killing were restored by blocking the interactions with 2B4 and NTB-A (Hislop et al., 2010).

Inhibitory function of 2B4 is also observed in NK cells from SAP-deficient mice (Bloch-Queyrat et al., 2000). A recent study shows that this inhibitory pathway is further strengthened in mice lacking the three SAP family adaptors, SAP, EAT-2, and ERT (Dong et al., 2009). In the absence of the three SAP family adaptors, SLAM receptors, including 2B4, NTB-A/Ly108 Ly-9, CD84, and CRACC, become inhibitory receptors in NK cells that repressed other activating receptors like NKG2D. The biochemical mechanism of this inhibition is not clearly understood, but it may be explained by the blocking function of the SAP adaptor family. However, 2B4-mediated inhibition in the absence of SAP family adaptors seems to operate at least in part via SHIP-1 (Dong et al., 2009).

A proapoptotic role for SAP in T cells was also suggested (Snow et al., 2009; Nagy & Klein, 2010). Activation-induced cell death by TCR activation was defective in T cells of XLP-1 patients, although increasing TCR activation strength bypassed the defect. This pathway seems to be dependent on NTB-A. It is possible that this defect participates in the burst of $CD8^+$ T cells in response to EBV infection in XLP-1 patients.

Therefore, SAP appears to have (at least) dual functional roles in SLAM receptor signaling and function by acting both

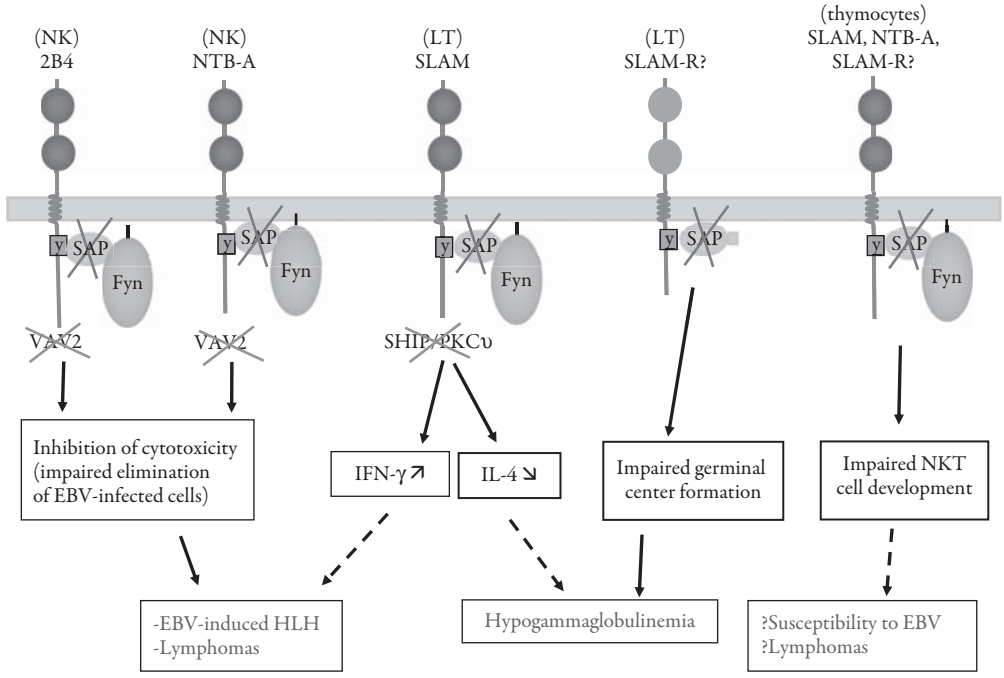

Figure 44.3 A model for defective SLAM receptor functions in the XLP pathophysiology. The loss of SLAM-R functions in the absence of SAP results in immune dysfunction contributing to the different clinical manifestations seen in XLP-1 patients. HLH and lymphoma are caused by dysfunction of SLAM receptors on $CD8^+$ T cells and NK cells that inhibit cell cytotoxicity responses and increase IFN-γ production. Similarly, T-helper follicular defects resulting in defective SLAM receptor function explain the hypogammaglobulinemia phenotype. Finally, it is possible that the lack of NKT cells participates in the high susceptibility to EBV infection, as these cells modulate immunity in a range of immunopathological conditions by their innate-like functions.

as an adaptor for the protein tyrosine kinase FynT and a competitor and/or a regulator of SH2-containing molecules that binds to SLAM receptors (Veillette et al., 2009).

SAP GENE MUTATIONS IN XLP-1 PATIENTS

More than 130 *SH2D1A* gene mutations have been identified in XLP-1 patients, including missense (M1I, M1T, A3S, Y7C, H8D, H8P, G16D, A22P, D26V, G27S, S28R, L31P, R32Q, R32T, D33Y, S34G, S34R, G39V, C42W, C42Y, G49V, T53I, T53R, Y54C, Y54H, R55L, R55P, S57P, E67D, E67G, T68I, I84T, F87S, E88P, D93G, Q99P, P101L, V102G), nonsense (R55X, Q58X, W64X, Y76X, N82FfsX103, I96X, Y100X, V102X, X129RfsX141), splice-site mutations, and micro- and macrodeletions (Alangari et al., 2006; Arico et al., 2001; Benoit et al., 2000; Booth et al., 2011; Brandau et al., 1999; Coffey et al., 1998; Erdös et al., 2005; Gilmour et al., 2000; Halasa et al., 2003; Hare et al., 2006; Honda et al., 2000; Hügle et al., 2004, 2007; Kanegane et al., 2012 ; Lappalainen et al., 2000; Lewis et al., 2001; Malbran et al., 2004; Morra et al., 2001a, 2001c; Nakamura et al., 2001; Nichols et al., 1998, 2005; Nistala et al., 2001; Pachlopnik Schmid J et al., 2011; Palendiar et al., 2011 ; Palendira et al., 2012, Parolini et al., 2000; Pasquier et al., 2005; Sayos et al., 1998; Soresina et al., 2002; Sumazaki et al., 2008; Sumegi et al., 2000; Tabata et al., 2005; Yin et al., 1999a). At present, the R55X mutation has been identified in at least 20 unrelated XLP-1 patients and seems to be the most prevalent mutation causing XLP-1 (Arico et al., 2001; Bottino et al., 2001; Brandau et al., 1999; Coffey et al., 1998; ; Hügle et al., 2004; Lappalainen et al., 2000; Lewis et al., 2001; Morra et al., 2001c; Nakajima et al., 2000; Parolini et al., 2000, 2002; Sumazaki et al., 2001 ; Sumegi et al., 2000). In one patient, no *SH2D1A* gene mutation was found in the coding regions as well as in the 500 bases upstream of the ATG, although SAP expression was undetectable in T-cell blasts derived from the patient (Verhelst et al., 2007). Therefore, it is very likely that mutations in a cis regulatory element distal from the *SH2D1A* gene or in one of its three introns could also predispose for XLP-1.

An *SH2D1A* gene mutation registry (SH2D1Abase) has been established, which is freely accessible through the Internet at http://www.uta.fi/imt/bioinfo/SH2D1Abase/ (Lappalainen et al., 2000). So far, no correlation between *SH2D1A* gene mutations and the clinical phenotype has been found. Identical mutations (e.g., the R55X mutation) may manifest different clinical phenotypes even within the same family (Brandau et al., 1999; Coffey et al., 1998; Lappalainen et al., 2000; Morra et al., 2001a; Sayos et al., 1998; Sumegi et al., 2000). Detectable mutations in the *SH2D1A* gene have been demonstrated in approximately 50 to 80 percent of patients with the XLP-1 phenotype (Brandau et al., 1999; Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998; Sumegi et al., 2000).

Most of the missense mutations of SAP result in a marked decrease in protein stability. However, Morra et al. (2001d) identified several mutations (R32Q, C42W, T53I, and T68I) with detectable expression that resulted in structural changes affecting interaction with the SLAM family receptors, SLAM, Ly-9, 2B4, and CD84 in a distinct fashion. Furthermore, most

SAP mutants caused by amino acid substitutions studied thus far (i.e., Y7C, S28R, L31P, R32Q, T53I, Y54C, R55L, E67D, T68I, F87S, G93D, Q99P, P101L, V102G) exhibited significantly reduced binding to the FynT SH3 domain (Li et al., 2003a, 2003b).

SH2D1A gene mutations have not been detected in any of 62 cell lines derived from sporadic Burkitt's lymphoma or in the peripheral blood of male patients with sporadic Burkitt's lymphoma or Hodgkin's disease, thus demonstrating that the *SH2D1A* gene does not play an important role in the development of (sporadic) Burkitt's lymphoma (Parolini et al., 2002; Yin et al., 1999b).

ANIMAL MODELS

The XLP-1 SCID-Hu Mouse Model

Severe combined immunodeficient (SCID) mice engrafted with peripheral blood lymphocytes (PBLs) from XLP-1 patients as well as from healthy seropositive controls readily developed EBV-induced oligo- or polyclonal lymphoproliferative disease regardless of the immunocompetence of the donors (Purtilo et al., 1991a). These lesions expressed all EBV-latent antigens (EBNA1-6, LMP), regardless of whether the SCID mice had been engrafted with PBLs from XLP-1 patients or from healthy controls. Engrafted PBLs of both groups also did not differ in their immunoglobulin production (IgG, IgA, and IgM). These findings suggest that the B cells of XLP-1 patients are functionally normal and exhibit no primary defect.

Graft-versus-host disease (GVHD) developed in 6 of 10 SCID mice engrafted with PBLs from five normal donors (EBV seropositive, n = 2; EBV seronegative, n = 3), but in none of 9 mice engrafted with PBLs from three XLP-1 males (EBV seropositive, n = 2; EBV seronegative, n = 1). These findings may be taken as tentative evidence for a primary T-cell deficiency in XLP-1.

The SAP-Deficient Mouse

Three different SAP-deficient mice have been obtained with a targeted disruption of the first exon of the *sh2d1a* gene (Wu et al., 2001) or with a *sh2d1a* gene mutation coding for SAP T68I (Czar et al., 2001) or with a partial deletion of intron 1 and exon 2 (Yin et al; 2003). To date, SAP-deficient mice have been shown to recapitulate most of the phenotypes found in XLP-1 patients, including enhanced $CD8^+$ and Th1 responses to viral infection, defects in NK-cell cytotoxicity response, hypogammaglobulinemia, decreased switched memory B cells, and lack of NKT cells (Calpe et al., 2008; Schwartzberg et al., 2009; Veillette et al., 2007, 2009). Importantly, several of these defects were found in Fyn-deficient mice, revealing the crucial role of the adaptor function of SAP. Further studies with mice expressing a SAP mutant protein, the SAP R78A with the arginine 78 mutated to alanine, which abolished the binding to the SH3 domain of Fyn, have confirmed the observations made in Fyn-deficient mice. The recent generation of different mice lacking single receptor of the SLAM family has further helped to define the role of each SLAM receptor in SAP-dependent lymphocyte functions. However, it is clear from these studies that there is redundancy, and several members of the SLAM family may cooperate to enable SAP to regulate T- and NK-cell functions.

In early studies, mice deficient in SAP were infected with lymphocytic choriomeningitis virus (LCMV), a virus that elicits strong and well-defined immune responses, or with murine gammaherpesvirus-68 (Wu et al., 2001; Yu et al., 2003). Dramatically increased numbers of virus-specific $CD8^+$ T cells with greater IFN-γ production capacity were found in the spleen and the liver of SAP-deficient animals. Confirming these findings, in vitro studies showed that $CD8^+$ T cells from SAP-deficient animals exhibit enhanced IFN-γ production and proliferation in response to TCR stimulation (Chen et al., 2005; Wu et al., 2001). This increase of $CD8^+$ T-cell numbers and function is apparently caused by decreased activation-induced cell death (Chen et al., 2007). When chronically infected by LCMV, SAP-deficient mice exhibited a severe immunopathology characterized by weight loss, lymphopenia in lymphoid organs, and death (Crotty et al., 2006). These signs resemble those associated with the fulminant infectious mononucleosis found in XLP-1 patients. These observations are reminiscent of findings reported in XLP-1 patients when infected by EBV. Importantly, depletion of $CD8^+$ T cells improved the pathology of SAP-deficient mice, indicating that exacerbated $CD8^+$ T-cell responses play a crucial role and implying that SAP is inhibitor of Th1 responses. These defects are accompanied by defective antibody production to LCMV and gammaherpesvirus-68 (Crotty et al., 2003; Yin et al., 2003).

Several studies have clearly established that SAP-deficient $CD4^+$ T cells produce low amounts of T-helper 2 (Th2) cytokines IL-4 and IL-13 and exhibit decreased ICOS and elevated CD40L expression in response to TCR stimulation (Cannons et al., 2004, 2006; Czar et al., 2001; Davidson et al., 2004; Wu et al., 2001). The role of SAP in Th2 cytokine production is dependent on the SLAM receptor, as SLAM-deficient mice have a similar defect to that found in SAP- and Fyn-deficient mice (Cannons et al., 2004; Davidson et al., 2004). However, partial Th2 cytokine defects were noticed in vitro with Ly108- and Ly9-deficient $CD4^+$ T cells, suggesting that several SLAM receptors cooperate to regulate $CD4^+$ T-cell function (Graham et al., 2006; Howie et al., 2005).

SAP-deficient mice generate only a short-lived IgG antibody response following LCMV infection, whereas the production of LCMV-specific, long-lived plasma cells and memory B cells was nearly absent (Crotty et al., 2003). These findings resemble the inability of XLP-1 patients to mount a sustained anti-EBV humoral immune response. Compromised antibody production is also observed when SAP-deficient mice are infected by the influenza virus and parasites such as *Toxoplasma gondii*, *Schistosoma mansoni*, and *Leishmania major* (Cannons et al., 2006; Chen et al., 2005; Czar et al., 2001; Kamperschroer et al., 2008; Wu et al., 2001) or when mice are immunized with T-cell–dependent protein antigens (Davidson et al., 2004; Hron et al., 2004; Morra et al., 2005). This altered antibody production was associated with defects in B-cell isotype switching, generation of memory B cells and

long-lived plasma cells, as well as a severe block in germinal-center formation (Al Alem et al., 2005; Crotty et al., 2003; Hron et al., 2004; Morra et al., 2005; Veillette et al., 2008).

Implication of T-helper defects in altered humoral immunity in SAP-deficient animal was first demonstrated by adoptive transfer experiments in SAP-deficient mice (Cannons et al., 2006; Crotty et al., 2003; Kamperschroer et al., 2008; Morra et al., 2005). These findings were confirmed by the generation of conditional SAP-deficient mice in which depletion of SAP in T cells leads to a lack in germinal-center formation and a marked decrease in switched memory B cells (Veillette et al., 2008). It was further shown that in the absence of SAP, follicular T-helper cells have a reduced ability to form stable contacts with B cells (Qi et al., 2008). This function of SAP seems to involve at least two SLAM-R, CD84 and Ly-108, which allowed sustained T-cell/B-cell contacts. Thus, abnormal expression of B-cell help molecules by T-helper cells and/or diminished Th2 cytokine production observed in SAP-deficient T cells seems not to account for the humoral defect in SAP-deficient mice as earlier suggested. The role of SAP in humoral responses does not require Fyn, as Fyn-deficient mice have normal antibody production (Cannons et al., 2006; McCausland et al., 2007).

Similar to findings in XLP-1 patients, impaired ability of 2B4 to enhance NK-cell cytotoxicity is present in SAP-deficient or in Fyn-deficient mice (Bloch-Queyrat et al., 2005). Ly-108 and Ly-9 have also the capacity to activate cell cytotoxicity in NK cells in a SAP-dependent manner (Dong et al., 2009). These studies have revealed an essential role of SLAM receptors for NK-cell surveillance of hematopoietic cells.

SAP-deficient mice, similarly to XLP-1 patients, completely lack NKT cells (Chung et al., 2005; Nichols et al., 2005; Pasquier et al., 2005). In SAP-deficient animals, the development of NKT cells is blocked at the positive selection step in the thymus. The phenotype of SAP-deficient mice resembles that of Fyn-deficient mice. However, the role of SAP in NKT-cell ontogeny seems not totally dependent on Fyn, as the development of iNKT cells in mice expressing the R78A SAP is only partially impaired (Nunez-Cruz et al., 2008; Pasquier et al., 2005). Analysis of SLAM- and Ly-108-deficient thymocytes in mixed-chimeras experiments revealed that these two SLAM receptors cooperate to deliver appropriate signals required for NKT-cell development (Griewank et al., 2007).

The understanding of the functions of SAP and the SLAM family receptors has shed light on the pathophysiology of XLP-1. Based on these findings, most of the clinical features of XLP-1 are currently understood to be the result of dysfunctions of the SLAM receptors in the absence of SAP (Fig. 44.3).

X-LINKED LYMPHOPROLIFERATIVE DISEASE TYPE 2 (XLP-2) DUE TO DEFECTS OF XIAP

In 2006, mutations in the gene *XIAP* (X-linked inhibitor of apoptosis) were identified in a cohort of XLP patients without detectable mutations in the *SH2D1A* gene and with normal expression of SAP (Rigaud et al., 2006) (Fig. 44.1b). In these patients, *XIAP* (also known as *BIRC4*) gene defects are responsible for a novel type of X-linked lymphoproliferative syndrome designated as XLP type 2 (XLP-2) (MIM #300635). These findings have been confirmed with the identification of additional patients (Doshi et al., 2008; Filipovich, 2010; Marsh et al., 2010a, 2010b; Pachlopnik Schmid et al., 2011; Worthey et al., 2011; Zhao et al., 2010).

One may also consider that earlier papers reporting XLP before the identification of *SH2D1A* as the first defective gene causing XLP likely included patients with defects in *XIAP*. Clinical similarities and differences in patients with XLP-1 and XLP-2 have recently been summarized based on a retrospective analysis of a cohort of 63 patients whose molecular diagnosis had been confirmed at the Necker Children's Hospital in Paris (Pachlopnik Schmid et al., 2011).

CLINICAL PHENOTYPES AND PATHOLOGICAL MANIFESTATIONS

Similarly to *SH2D1A* deficiency (XLP-1), *XIAP* deficiency (XLP-2) is mainly characterized by a peculiar susceptibility to EBV infection (Pachlopnik Schmid et al., 2011; Rigaud et al., 2006; Zhao et al., 2010) (Table 44.1). However, XLP-1 and XLP-2 can be distinguished by several specific clinical features (Rigaud et al., 2006). The differences and similarities of XLP-1 and XLP-2 have been analyzed recently in more details in two cohorts of 33 and 30 patients with XLP-1 and XLP-2, respectively (Pachlopnik Schmid et al., 201). Survival rates of SAP- and XIAP-deficient patients did not differ, and HLH (XLP-1, 55 percent; XLP-2, 76 percent) and hypogammaglobulinemia (XLP-1, 67 percent; XLP-2, 33 percent) were observed in both groups and apparently did not differ significantly. Infection by EBV was predominantly the common trigger of HLH in both groups (XLP-1, 92 percent; XLP-2, 83 percent). The mean ages at the first HLH occurrence are similar, but HLH with lethal outcome was more frequent in XLP-1. In contrast to XLP-1 patients, 30 percent of whom developed lymphoma, XLP-2 patients do not suffer from lymphomas but have an increased risk to develop chronic hemorrhagic colitis resembling inflammatory bowel disease (IBD) or Crohn's-like disease, a complication observed in 17 percent of XLP-2 patients. Confirming that XIAP may represent a novel IBD gene susceptibility, a recent report identified by whole-exome sequencing a deleterious missense mutation in *XIAP* in a 15-month-old boy with severe Crohn's-like disease (Worthey et al., 2010). One other characteristic feature of XLP-2 is splenomegaly (XLP-1, 7 percent; XLP-2, 87 percent); in several cases this has been the first clinical manifestation of the XLP-2 condition (Doshi et al., 2008; Pachlopnik Schmid et al., 2011; Rigaud et al., 2006; Horn et al, 2011). Splenomegaly is mostly recurrent and associated with cytopenia and fever. Based on histopathology, it very likely represents minimal forms of HLH (Pachlopnik Schmid et al., 2011).

In 2009, a cohort of 10 American patients with XIAP deficiency was reported, and interestingly, EBV infection was not found to be the predominant trigger of HLH (30 percent)

(Marsh et al., 2010b). In one patient, HLH was concomitant with a CMV infection, while in the others HLH occurred without a known viral trigger. Thus, minimal forms of HLH and/or full-blown HLH can occur in XLP-2 patients without a clearly identified pathogen trigger.

So far, no cases of lymphoma have been reported in patients with XIAP deficiency. Although the possibility of developing lymphoma has not been formally excluded in XIAP-deficient patients, this complication seems unlikely because XIAP is an anti-apoptotic molecule, and studies using XIAP inhibitors have provided evidence that XIAP represents a potent target for the treatment of cancer (Schimmer et al., 2006). Although 33 percent of XIAP-deficient patients were found to have hypogammaglobulinemia, this complication, in contrast to XLP-1, seems to be transient, as two patients recovered normal levels of Ig (Pachlopnik Schmid et al., 2011). It is possible that in the absence of XIAP, hypogammaglobulinemia develops as a consequence of the HLH and/or secondary to the immunosuppressive treatments, which might affect transiently B cells. Also, most of the XIAP-deficient patients have normal Ig-isotype-switched memory B cells, unlike SAP-deficient patients (S. Siberil and S. Latour, unpublished observations).

Analysis in two independent cohorts of CVID patients failed to detect mutations in *XIAP*, indicating there is no overlapping phenotype (Salzer et al., 2008; Christelle Lenoir, Sylvain Latour, and Ioannis Theodorou, unpublished data).

MOLECULAR BASIS

The identification of the XLP-2 gene defect was performed by linkage studies and positional cloning. A unique region at Xq25 cosegregating with the disease in three XLP families was first identified and further refined (Rigaud et al., 2006). Notably, both XLP-2 (*XIAP*) and XLP-1 (*SH2D1A*) genes are located in the same gene locus in Xq25, and the two genes are only separated by a physical distance of 2.5 Mb. The human *XIAP* (also named *BIRC4*, for baculoviral IAP repeat [BIR]-containing protein 4) gene consists of six exons spanning 55 kb that encodes for cytoplasmic protein of 497 amino acids (Fig. 44.1b). XIAP belongs to the family of inhibitors of apoptosis proteins (IAPs) and in humans comprises eight members, with IAP-1 and IAP-2 being the closest XIAP-related proteins. XIAP consists of three BIR domains (BIR1, BIR2, and BIR3) that mediate protein–protein interactions, one ubiquitin binding domain (UBA), and a C-terminal RING domain with an ubiquitin protein ligase (E3) activity (Gyrd-Hansen et al., 2008; Vaux et al., 2005).

XIAP is ubiquitously expressed. In hematopoietic cells, the expression of XIAP is comparable in the different cell subpopulations tested, including $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, dendritic cells, NK cells, and NKT cells (Marsh et al., 2010a; Rigaud et al., 2006).

FUNCTION OF THE XIAP PROTEIN

XIAP is a potent physiological inhibitor of caspases 3, 7, and 9 (Eckelman et al., 2006). The substrate-binding clefts of caspases 3 and 7 are directly blocked by the interaction with the BIR2 domain of XIAP. By contrast, inhibition of caspase 9 is mediated by the BIR3 domain that sterically hinders caspase 9 dimerization and its subsequent activation. The anti-apoptotic activity of XIAP is regulated by Smac (also known as DIABLO) and Omi. Following apoptotic stimuli, the protein Smac/DIABLO is released from the mitochondria and binds to the BIR2 and BIR3 domains of XIAP, relieving by this way caspase inhibition. In proliferating T cells, XIAP inhibits caspases 3 and 7 (Paulsen et al., 2008).

In addition to its anti-apoptotic role, XIAP is also involved in multiple signaling pathways, including copper metabolism, activation of the NFκB and MAP kinase pathways, and TGF-β (type 1) receptor and BMP (type 1) receptor signaling (Dubrez-Daloz et al., 2008; Galbán et al., 2010). XIAP induces NFκB and the MAP kinase activation via its BIR1 domain that mediates dimerization and interacts with the adaptor protein TAB1. TAB1 directly activates TAK1, a MAP kinase kinase kinase (MAPKKK), which in turn activates the MAP kinase cascade and the inhibitor of IκB kinase (IKK). However, the RING and the UBA domains of XIAP also contribute to the activation of NFκB. The UBA domain in IAP proteins enables them to bind to Lys 63-linked polyubiquitylated proteins. Lys 63-linked polyubiquitylation is involved in nondegradative signaling processes such as activation of NFκB. A recent report provides evidence that XIAP is also involved in NFκB activation by NOD1 and NOD2 intracellular receptors, members of the NOD-like receptor family (NLR) involved in the sensing of pathogens. XIAP was found to associate via its BIR2 domain to the receptor-interacting protein kinase RIP2, an inducer of NFκB that interacts with NOD1 and NOD2 (Krieg et al., 2009).

LABORATORY FINDINGS AND IMMUNOLOGICAL STUDIES IN XLP-2 PATIENTS

The clinical manifestations of XLP-1 and XLP-2 are similar, suggesting that they share common defects. In $CD8^+$ T cells and NK cells, SAP deficiency results in abnormal function of the SLAM-related receptor 2B4. By contrast, IL-2–activated NK cells from XIAP-deficient patients show intact 2B4-mediated cytotoxicity, and their overall cytotoxicity function is normal when measured against K562 target cells (Marsh et al., 2010b; Rigaud et al., 2006). Cytotoxicity and degranulation of $CD8^+$ T cells was also found to be normal in the absence of XIAP (C. Synaeve and S. Latour, unpublished observation). XIAP-deficient patients can also display low but detectable numbers of iNKT cells (0.005–0.02 percent of total T lymphocytes) in the blood; in contrast, SAP-deficient patients have almost no detectable iNKT cells (<0.005 percent) (Marsh et al., 2009; Rigaud et al., 2006).

In accordance with the anti-apoptotic role of XIAP, T-cell blasts from XIAP-deficient patients exhibit augmented apoptosis in response to cross-linking of CD95 or the Fas-related receptor TRAIL-R (Rigaud et al., 2006). Similarly, activation-induced cell death following anti-CD3 stimulation, which is known to be CD95-dependent, is markedly increased in XIAP-deficient T-cell blasts. The increased apoptosis in response to

CD95 signaling is not restricted to T cells as it is also observed in EBV-transformed B cells. This excess of apoptosis is caused by the lack of XIAP, as reconstitution experiments with wild-type XIAP restored normal levels of apoptosis in T-cell blasts and EBV-transformed B cells from XIAP-deficient patients.

Although XIAP-deficient lymphocytes are more prone to apoptosis in vitro, XLP-2 patients seem not to develop T-, B-lymphopenia, with the exception of iNKT cells. In contrast to conventional T cells, iNKT cells might be noticeably sensitive to activation-induced apoptosis and, consequently, more dependent on XIAP for their survival. However, in vivo XIAP is probably also involved in the survival of other leukocytes, as a nonrandom X-chromosome inactivation within leukocytes of XIAP-mutated female carriers is observed, indicating that cells expressing the wild-type XIAP allele have been preferentially selected (Rigaud et al., 2006). These observations were confirmed at the protein level in a recent study (Marsh et al., 2010a). The analysis of NOD2 responses in cells from a XIAP-deficient patient with chronic colitis has revealed a decreased responsiveness to NOD2 ligands, confirming the role of XIAP in NOD receptor signaling (Worthey et al., 2010).

XIAP GENE MUTATIONS IN XLP-2 PATIENTS

To date, more than 27 mutations in *XIAP* have been identified, including missense (R166I, C203Y, G188E, W173G, C230Y, T470S, P482R, I494N), nonsense (E118X, R222X, R238X, Q171X, S253X, Q333X, R381X, Q104X, G466X), and frameshift mutations (E99KfsX129, D130GfsX140, W217CfsX27, K299fsX307, I339fsX348, N341YfsX7, S347Lfs5X, I397FfsX414, E434AfsX457, P482fsX508) and deletions (Exon 2, Del Exons 1–5, Del Exon 6) (Doshi et al., 2008; Fillipovitch et al., 2010; Horn et al., 2011; Marsh et al., 2010b; Pachlopnik Schmid et al., 2011; Rigaud et al., 2006; Yang et al., 2012; Zhao et al., 2010) (Fig. 44.1b). Several of these mutations allow a decreased or normal expression of full-length or truncated protein (Del Exon 6, G466X, T470S, P482R, G188E, E434AfsX457). It is possible that these mutants behave as hypomorphic mutants with less severe phenotype and/or incomplete penetrance. The mothers of XLP-2 patients were found to be asymptomatic heterozygous carriers.

ANIMAL MODELS

XIAP-Deficient Mice

XIAP-deficient mice have been obtained by deletion of exon 1 of *xiap* or deletion of both exon 1 and 2 of *xiap* (Harlin et al., 2001; Olayioye et al., 2005). XIAP-deficient mice were originally reported with no major phenotypic abnormalities, including the lack of obvious abnormal apoptotic phenotypes in cells of the immune system. However, a few specific defects have been identified. Sympathetic neurons and postmitotic cardiomyocytes from XIAP-deficient mice are more sensitive to cytochrome c-induced cell death. XIAP deficiency also leads to delayed lobuloalveolar development in the mammary gland, even though this aberrant phenotype does not result from altered apoptosis and/or proliferation. More recently, XIAP-deficient mice were reported to be more susceptible to infections with *Chlamydophila pneumoniae* and *Listeria monocytogenes* (Bauler et al., 2008; Prakash et al., 2010). In these conditions, decreased cell survival and abnormal cytokine production by macrophages, presumably via TLR-2 and NOD2 receptors, were observed. Importantly, these mice have normal NKT-cell development and normal B-cell function and humoral responses, unlike SAP-deficient mice (Rigaud et al., 2006; Rumble et al., 2009; S. Siberil and S. Latour, unpublished data). Thus far, it is not clear whether XIAP-deficient mice represent a valid model for XLP-2.

The mechanisms underlying the pathophysiology of XLP-2 are not understood and remain to be elucidated. Until now, the defect in NKT cells has been the only immune abnormality shared by XIAP and SAP deficiency, and it is possible that absence or low numbers of iNKT cells contribute in part to the high susceptibility to EBV infection in XLP (Latour et al., 2007). However, the findings that XIAP is involved in NOD2 function may explain the increased risk of XIAP-deficient patients to develop chronic colitis, as NOD2 is one susceptibility gene for Crohn's disease.

DIAGNOSTIC CONSIDERATIONS (XLP-1 AND XLP-2)

The diagnosis of XLP-1 and XLP-2 is based on clinical, immunological, and molecular findings (Pachlopnik Schmid et al., 2011) (Table 44.2) and can only be confirmed by mutation analysis. Prenatal diagnosis or the evaluation of a heterozygous status in (clinically healthy) women is possible by direct mutation analysis of the *SH2D1A* or *XIAP* gene and/or its RNA (Hügle et al., 2004; Shinozaki et al., 2002), or indirectly (in informative families) by RFLP analysis with X-chromosomal markers flanking both *SH2D1A* and *XIAP* genes proximally and distally (Fig. 44.4) (Mulley et al., 1992; Rigaud et al., 2006; Skare et al., 1992; Schuster et al., 1993, 1994, 1995). Furthermore, SAP or XIAP expression can be easily measured by FACS analysis in $CD3^+$ or $CD8^+$ T cells

Table 44.2 **CRITERIA FOR DIAGNOSIS OF X-LINKED LYMPHOPROLIFERATIVE DISEASE**

Definitive diagnosis of XLP-1 or -2
Male patient with one or more XLP phenotypes plus SAP or XIAP deficiency (i.e., mutations in the *SH2D1A* or *XIAP* gene, absent mRNA and/or absent protein expression of the *SH2D1A* gene or *XIAP* in lymphocytes)*
Probable diagnosis of XLP-1 or -2
One or more XLP phenotypes in a male patient *plus* one or more XLP phenotypes in at least one of his brothers, maternal cousins, uncles, or nephews (*SH2D1A* and *XIAP* gene status not known)
Suggestive diagnosis of XLP-1 or -2
One or more XLP phenotypes in a single male family member (*SH2D1A* and *XIAP* gene status not known)

*Presymptomatic diagnosis of XLP-1 or 2 is also definitive in an (initially) healthy male carrying a *SH2D1A* or *XIAP* gene mutation known to cause XLP-1 or -2.

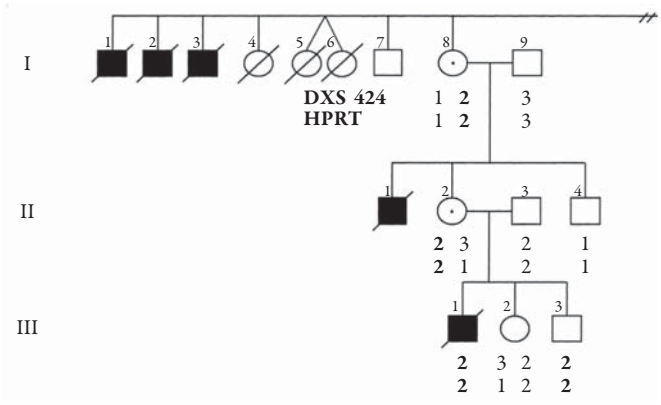

Figure 44.4 Pedigree of a Spanish XLP-1 family with haplotypes of polymorphic markers DXS424 (proximal to *SH2D1A*) and HPRT (distal to *SH2D1A*). Male subjects I-1 to I-3, II-1, and III-1 (black symbols) died of fulminant infectious mononucleosis during early infancy. Haplotype 2-2 co-segregated with XLP-1 (Brandau et al., 1999). In the index patient 3 (III-1), but also in his healthy brother, a nonsense mutation of the *SH2D1A* gene (Y100X) was identified (Brandau et al., 1999). Subjects I-8 and II-2, but not III-2, were shown to be heterozygous carriers for the Y100X mutation.

and NK cells or Western blotting in PBMC or PHA blasts (Marsh et al., 2010a; Rigaud et al., 2006; Shinozaki et al., 2002; Tabata et al., 2005). For SAP deficiency, the carrier status can be easily evaluated by FACS, revealing a bimodal expression of SAP in $CD8^+$ T cells and NK cells. For the carrier status of a XIAP deficiency, determination by FACS is not indicated, as the XIAP wild-type allele is preferentially expressed in hematopoietic cells of the carrier, making detection of a clear bimodal expression of XIAP difficult (Marsh et al., 2010a). Because iNKT cells are nearly absent in XLP-1 patients, the quantification of iNKT cells, together with clinical data and family history, may facilitate the diagnosis of XLP-1. However, for the diagnosis of XLP-2 it might not be relevant, as XLP-2 patients can have normal numbers of iNKT cells (Marsh et al., 2009). To validate missense mutations of XIAP that retain XIAP expression, activation-induced cell death of PHA-T cell blasts upon TCR/CD3 stimulation can be performed. When adequately done in parallel with control cells from healthy donors, it reveals a significant and reproducible excess of apoptosis in T-cell blasts of the patient.

DIFFERENTIAL DIAGNOSIS

While there are some differences in patients with XLP-1 and XLP-2 (Pachlopnik Schmid et al., 2011), the clinical phenotypes of these two syndromes share similarities with other disorders that need to be excluded. These are discussed below. Sporadic fatal infectious mononucleosis occurs in approximately 1 in 3,000 cases of infectious mononucleosis (Purtilo et al., 1992), with a median age of 5.5 years (fatal infectious mononucleosis in XLP-1 occurs at a median age of 2.5 years) and a male/female ratio of approximately 1:1 (Mroczek et al., 1987b). Presenting signs and symptoms are similar to those seen in XLP-1 patients with fatal infectious mononucleosis. Other XLP-1 phenotypes (dysgammaglobulinemia, lymphoma) are absent. In 25 males with sporadic fatal infectious mononucleosis studied to date, no mutations within the *SH2D1A* gene have been found (Sumegi et al., 2000).

Familial hemophagocytic lymphohistiocytosis (FLH) is a group of autosomal-recessive disorders characterized by HLH that resembles HLH/VAHS observed in male XLP-1 and XLP-2 patients (see Chapter 53). Male and female patients with HLH harbor mutations in genes encoding molecules involved in the cytotoxic functions of $CD8^+$ T cells and NK cells. These include mutations in the perforin1, MUNC13–4, MUNC18-2 (STXBP2), syntaxin 11, LYST, and RAB27 genes. Similarly to XLP-1 and XLP-2 patients, affected children may present with fever, splenomegaly, hepatomegaly, pancytopenia, coagulation abnormalities, neurological abnormalities, and high serum concentrations of IFN-γ and TNF-α. In contrast to patients with XLP, the hemophagocytic syndrome in these patients is not particularly associated with EBV infection, and the trigger of HLH is unknown in most of the cases. The onset of the disease is earlier than in XLP-1 and XLP-2, but, importantly, in late-expressing forms of FHL, EBV infection is often one of the triggers of HLH (Katano et al., 2004). Conversely, a few male patients with an HLH-like disease not triggered by EBV have been found to have mutations in the *SH2D1A* gene (Arico et al., 2001; Halasa et al., 2003) or *XIAP* gene (Marsh et al., 2010b; Rigaud et al., 2006).

An autosomal-recessive lymphoproliferative syndrome with hepatosplenomegaly associated with recurrent EBV infections has been recently described in two sisters of a consanguineous Turkish family who exhibited a homozygous missense mutation (R335W) in the gene encoding the IL-2–inducible T-cell kinase (ITK) (Huck et al., 2009). The girls died at age 5 and 10 years, respectively, after developing severe immune dysregulation and therapy-resistant EBV-positive B-cell proliferation identified as EBV-associated Hodgkin's lymphoma. iNKT cells were absent in both patients, a shared feature of XLP-1. However, no signs of HLH have been reported. In 11 further patients with ITK deficiency reported so far additional mutations in the ITK gene were identified: R29H, D500T, F501L, M503X, R265X, Y588X. Clinically these patients presented also with massive EBV+ B cell lymphoproliferation (EBV + LPD, EBV + Hodgkin ′s lymphoma) and hypogammaglobulinemia (Linka et al., 2012; Mansouri et al., 2012; Stepensky et al., 2011).

A similar autosomal-recessively inherited disorder with severe CD4+ lymphopenia and EBV-associated lymphoproliferation was recently identified in three siblings with homozygous Coronin-1A deficiency (Moshous et al., 2013)

EBV-associated lymphoproliferative disorder (EBV+ LPD) with or without HLH, malignant lymphoma or absence of overt clinical phenotype have been recently found also in nine patients lacking functional CD27 (van Monfrans et al., 2011; Salzer et al., 2013).

Chronic active EBV (CAEBV) infection involving mostly T or NK cells is characterized by a persistent severe mononucleosis-like illness (lasting for months or years) with additional unusual clinical features such as hypersensitivity to mosquito bites (43 percent), calcifications in basal ganglia (18 percent),

hydroa vacciniforme (14 percent), hemophagocytic syndrome (21 percent), coronary artery aneurysms (21 percent), and malignant lymphoma (16 percent) (Kimura et al., 2001). Patients with CAEBV have a high EBV load in their peripheral blood ($>10^{2.5}$ copies/μg DNA). In this syndrome, EBV infects predominantly T and/or NK cells (Kasahara et al., 2001; Kimura et al., 2001). In 18 male CAEBV patients studied, no mutations of the *SH2D1A* gene were found (Kimura et al., 2001; Sumazaki et al., 2001). However, one CAEBV patient exhibited a mutated perforin gene (Katano et al., 2004).

Common variable immunodeficiency (CVID) should also be considered in the differential diagnosis (see Chapter 27). The characteristic feature of this rather heterogeneous group of patients is a decrease in all or at least two major immunoglobulin isotypes and an impaired antibody response. In the majority of CVID patients B cells are present in normal numbers. However, these cells are unable to differentiate into antibody-producing plasma cells, possibly due to a primary T-cell abnormality. In a small subset of CVID patients, mutations in the *ICOS* (Grimbacher et al., 2003) or *TACI* genes (Castigli et al., 2005; Salzer et al., 2005) have been identified. In general, CVID occurs in a sporadic manner. Other than the antibody deficiency, there is little similarity to XLP-1. However, a subset of male XLP-1 patients may present with a CVID-like disease (Gilmour et al., 2000; Morra et al., 2001c; Nistala et al., 2001; Soresina et al., 2002).

LABORATORY FINDINGS IN XLP-1 FEMALE CARRIERS

None of the female carriers of the XLP-1 gene studied thus far developed any of the XLP-1 phenotypes. Subtle immunological abnormalities such as hypogammaglobulinemia and elevated IgA and/or IgM levels have been found in a few carrier females (Grierson and Purtilo, 1987; Morra et al., 2001c; Sakamoto et al., 1982). Forty-five of 56 female carriers (80 percent) have been shown to exhibit an abnormal serological response to EBV infection (Grierson and Purtilo, 1987). No data are available for female carriers of the XLP-2 gene.

However, EBV serology alone is not a reliable diagnostic finding in the predictive evaluation of female carriers, since transiently elevated serum antibodies against VCA and EA of the IgM, IgG, or IgA class are also found in normal subjects with a recent primary EBV infection. Carrier females of XLP-1 have a random X-chromosome inactivation pattern in their B and T cells (Conley et al., 1990; Nichols et al., 2005). However, the X-chromosome inactivation pattern in NKT cells of female XLP-1 carriers is skewed (Nichols et al., 2005). In carrier females of XLP-2, the X-chromosome inactivation is skewed in all hemopoietic cells tested, including T, B cells and myeloid cells in which the wild-type allele is preferentially expressed (Marsh et al., 2010a; Rigaud et al., 2006).

At present, direct mutation analysis of the *SH2D1A* or *XIAP* (Birc4) gene or indirect genotype analysis (in informative families) is the only reliable means to determine if a female at risk is a carrier for XLP-1 or XLP-2.

X-LINKED LYMPHOPROLIFERATIVE DISEASE TYPE (XLP-3) DUE TO DEFECTS IN *MAGT1*

In 2011, two unrelated families with a total of three affected males presenting with "idiopathic" CD4 lymphopenia and chronic EBV infection were found to have mutations in the magnesium transported gene, *MAGT1* (OMIM300715). The syndrome, X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia, has been named XMEN (Li et al., 2011). The patient of family 1 with a nonsense mutation in exon 3 of *MAGT1* (which has 10 exons) died at 45 years of age from chronic EBV-associated lymphoma. The second family had two affected brothers who suffered from recurrent infections, including chronic EBV infection. Both had inverted CD4:CD8 ratios, impaired thymic output of $CD4^+$ T cells, defective TCR-mediated activation in response to anti-CD3, but normal B-cell stimulation via the B-cell receptor or Toll-like receptor. While in normal T cells antigen receptor stimulation results in a rapid transient Mg^{2+} influx, MAGT1-deficient T cells fail to manifest a Mg^{2+} influx, leading to impaired responses to antigen receptor engagement, including defective activation of phospholipase Cγ1 and markedly impaired $C\alpha^{2+}$ influx in T but not B cells. Thus, *MAGT1* deficiency is the third X-linked condition associated with chronic EBV infection and lymphoma. However, in contrast to XLP-1 and XLP-2, affected males have normal numbers of NKT cells and HLH has not been reported.

TREATMENT AND PROGNOSIS

XLP-1 PATIENTS

Prophylactic Treatment of Males at Risk Prior to EBV Infection

There is evidence that maternal EBV-neutralizing antibodies may protect newborn infants, including XLP males, from primary EBV infection for 4 to 6 months (Biggar et al., 1978; Mroczek et al., 1987b). Regular intravenous infusions with immunoglobulins rich in EBV-neutralizing antibodies were therefore started in males at risk to prevent or lessen the effects of EBV infection. However, some boys succumbed to fulminant mononucleosis despite this therapy (Okano et al., 1990, 1991; Seemayer et al., 1995). In any case, all EBV-negative XLP-1 males with hypogammaglobulinemia should receive regular immunoglobulin substitutions (intravenously or subcutaneously) to prevent severe bacterial or viral infections.

Currently, the only means of preventing EBV- and non–EBV-related complications in later life is early transplantation of allogeneic hematopoietic stem cells (HSCT) (Arkwright et al., 1998; Booth et al., 2011; Gross et al., 1996; Hoffmann et al., 1998; Lankester et al., 2005; Pracher et al., 1994; Trottestam et al., 2009; Vowels et al., 1993; Williams et al., 1993; Ziegner et al., 2001). The age of the patient at the time of transplantation appears to be critical. Of eight XLP-1 patients who underwent stem cell transplantation, four boys less than 15 years of age were alive and well for more than 2 years after transplantation, whereas all four boys older

than 15 years of age at the time of transplantation died within 90 days of complications (Gross et al., 1996; Seemayer et al., 1995). Transplantation should be undertaken at the youngest possible age in all patients with HLH because of the poor outcome. Survival of patients with HLH undergoing HSCT is 50 percent but falls to 18.8 percent in untransplanted patients with HLH (Booth et al., 2011).

Gene therapy may become an option in the future. In the murine XLP-1 model it as already been shown that SH2D1A gene transfer into hematopoietic stem cells can correct the multiple immune defects seen in XLP-1 (Rivat et al., 2013).

TREATMENT OF XLP MALES DURING OR AFTER EBV INFECTION

During fulminant mononucleosis, the outcome of treatment with high-dose immunoglobulin, antiviral drugs such as acyclovir, immunosuppressive agents, and IFN-γ has been disappointing (Okano et al., 1990, 1991). Etoposide (VP-16), which has been effective in the treatment of lymphoma, leukemia, and hemophagocytic syndromes, has been shown to induce remissions in some XLP-1 males with fulminant mononucleosis, HLH, or aplastic crisis (Migliorati et al., 1994; Okano and Gross, 1996; Seemayer et al., 1995). One such patient showed rapid improvement of his clinical symptoms of fulminant mononucleosis for a short period after treatment with etoposide. Subsequently, after relapse and clinical deterioration, the boy underwent successful stem cell transplantation following conditioning with etoposide, busulfan, and cyclophosphamide (Pracher et al., 1994). In view of the unfavorable prognosis, a controlled clinical trial with etoposide, with or without stem cell transplantation, in patients with acute exacerbations of XLP-1 is warranted.

One XLP-1 patient with life-threatening EBV-associated HLH reported so far was finally successfully treated by initial intravenous immunoglobulin (IVIG) and high-dose corticosteroids followed by the institution of the HLH-2004 treatment protocol, which includes etoposide, dexamethasone, cyclosporin A, and intrathecal therapy with methotrexate and prednisolone. To reduce the EBV load in B cells, the patient additionally received anti-CD20 monoclonal antibody (rituximab) on day 1. For attenuating the EBV-induced hyperinflammatory state ("cytokine storm"), the patient received the tumor necrosis factor alpha (TNF-α) blocker etanercept (25 mg intravenously) on days 2 and 7 (Mischler et al., 2007).

Males with XLP-1 who develop hypogammaglobulinemia in response to EBV infection require regular intravenous (monthly) or subcutaneous (weekly) immunoglobulin replacement (Nistala et al., 2001) to prevent recurrent bacterial and viral infections. Despite immunoglobulin replacement therapy, other XLP-1 phenotypes such as aplastic anemia or lymphoma may develop in subsequent years (Purtilo, 1991).

XLP-1 patients suffering from malignant lymphomas may enter remissions of short duration after undergoing standard chemotherapy protocols (Seemayer et al., 1995). However, relapses are frequent and other XLP-1 phenotypes may develop. XLP-1 patients with active EBV infection and lymphoproliferative complications may be successfully treated with rituximab in combination with prednisone, acyclovir, and IVIG infusions (Malbran et al., 2004; Milone et al., 2006). Eventually, this procedure may also be useful in the treatment of B-cell lymphoma in XLP-1 patients.

XLP-2 PATIENTS

The therapeutic and prophylactic experiences in XLP-2 patients are still limited due to the low numbers of affected subjects studied and reported so far (Filipovich, 2010; Marsh et al., 2010b; Rigaud et al., 2006). However, XLP-2 patients should be treated like XLP-1 patients according to the HLH-2004 (or a similar) treatment protocol. Treatment with rituximab may be also successful. The indication for stem cell transplantation in XLP-2 is controversial. A multicenter international study of 19 XLP-2 patients with confirmed XIAP deficiency undergoing HSCT revealed poor outcomes. Of seven patients receiving myeloablative busulfan-containing conditioning, only one patient (14 percent) survived; most deaths were caused by transplant-related toxicity. Of the 11 patients receiving reduced-intensity conditioning, 6 (55 percent) are surviving at a median of 570 days following transplantation (Marsh et al., 2013). In contrast, by combining a minimal-intensity immunoablative conditioning regimen with a high stem-cell and T-cell dose graft, XIAP deficiency with uncontrolled HLH has been cured in a 2 year old boy (Worth et al, 2013). Interestingly, one patient with a chronic colitis/IBD was successfully transplanted; at the time of the report, the symptoms of his bowel disease had completely resolved (Worthey et al., 2010). Like XLP-1, XLP-2 patients with hypogammaglobulinemia should be given weekly (subcutaneously) or monthly (intravenously) immunoglobulin infusions.

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45.

DIGEORGE SYNDROME: A CHROMOSOME 22q11.2 DELETION SYNDROME

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The association of thymic aplasia with congenital hypoparathyroidism was initially noted by Lobdell in 1959 but wasn't recognized as a syndrome until 1965, when Dr. Angelo DiGeorge described a group of infants with congenital absence of the thymus and parathyroid glands (DiGeorge, 1965). Subsequently, facial dysmorphia and cardiac defects, specifically conotruncal malformations, were included in the spectrum of DiGeorge syndrome (DGS) (Conley et al., 1979). DGS (MIM 188400) is a heterogeneous disorder. However, cytogenetic and molecular studies have shown that deletion of chromosomal region 22q11.2 is the leading cause of DGS. In rare instances, DGS is seen in association with other chromosomal rearrangements, with exposure to teratogens such as retinoic acid, and with maternal diabetes (Lammer and Opitz, 1986; Wilson et al., 1993b).

DGS is a developmental disorder that occurs as a result of abnormal cephalic neural crest cell migration, differentiation, or signaling in the third and fourth pharyngeal arches during the fourth week of embryonic development. Kirby et al. (1983) demonstrated that removal of the premigratory cardiac neural crest cells in the chick embryo results in cardiac outflow tract anomalies similar to those seen in DGS. The cardiac neural crest cells have also been shown to be important in supporting the development of the glandular derivatives of the pharyngeal arches (Kirby and Bockman, 1984). The aortic arch anomalies have been duplicated in transgenic mice with haploinsufficiency due to a deletion of a region of mouse chromosome 16 (Df1) that is homologous to human chromosome 22q11 and contains the *Tbx1* gene encoding a transcription factor with "T-box" DNA-binding domain (Lindsay et al., 2001; Merscher et al., 2001). Jerome and Papaioannou (2001) produced a mouse knockout with a null mutation of *Tbx1*. The heterozygotes have cardiovascular anomalies seen in DGS, whereas the homozygotes are nonviable but display a DGS phenotype as embryos. In fact, the dose of T-box 1 transcription factor during embryonic development is critical for the phenotypic manifestations (Liao et al., 2004).

The gene *TBX1* is the primary gene responsible for DGS, although other genes in the region may contribute to the phenotypic variability. Confirming the importance of the human gene *TBX1* in human DGS, a few patients with classical features of DGS have now been identified with point mutations in *TBX1*. Mutations were also identified in patients with less classical manifestations (Yagi et al., 2003). A further consideration is that gene expression in the gene-rich DGS region of 22q11.2 may be influenced by a long-range regulatory element (Botta et al., 2001).

DGS was initially considered a rare sporadic disorder; however, recent studies suggest that the 22q11 deletion, seen in approximately 90 percent of DGS patients, may occur as frequently as once in 4,000 to 6,000 live births, affecting both sexes equally. Furthermore, acknowledgment of the phenotypic overlap with other genetic disorders that share the 22q11.2 deletion, such as velo-cardio-facial syndrome (VCFS, MIM 192430) and conotruncal anomaly face syndrome (CTAFS), has led to further expansion of the phenotype and a better understanding of the immune, endocrine, cognitive, neurological, and psychiatric problems arising in DGS patients. While most early studies evaluated patients with the classic features of DGS, recent studies have attempted to ascertain all patients with the deletion to better define the clinical spectrum. The immunodeficiency is comparable in all patients with the deletion regardless of the other clinical manifestations (Sullivan et al., 1998). Therefore, we will discuss all patients who carry the deletion under the nomenclature of DGS. It is important to remember that the nomenclature is still evolving, with the term *chromosome 22q11.2 deletion syndrome* being appropriately applied to patients in whom the deletion has been

Table 45.1 DIAGNOSTIC CRITERIA FOR DGS

DIAGNOSTIC CATEGORY	DESCRIPTION
Definitive	<500 CD3 T cells/mm^3 and two of the three following characteristics: Conotruncal cardiac defect Hypocalcemia requiring therapy for >3 weeks Deletion of chromosome 22q11.2
Probable	<1,500 CD3 T cells/mm^3 and deletion of chromosome 22q11.2
Possible	<1,500 CD3 T cells/mm^3 and at least one of the following: Cardiac defect Hypocalcemia requiring therapy for >3 weeks Dysmorphic facies or palatal anomaly

SOURCE: From the European Society for Immunodeficiencies.

confirmed. DGS is still used for both patients with 22q11.2 deletion and patients with the classical clinical triad of cardiac defects, immunodeficiency, and hypocalcemia but without a demonstrable deletion. Approximately 10 percent of patients with DGS will not have the standard chromosome 22q11.2 deletion; thus, the deletion is not absolutely required for the diagnosis of DGS. One scheme for categorizing patients with DGS is given in Table 45.1. The most accurate labels are the genetic descriptions, when applicable (i.e., chromosome 22q11.2 deletion syndrome) and the syndromic descriptor when the etiology is not known.

CLINICAL FEATURES

DGS has classically been characterized as a triad of clinical features: congenital cardiac defects, immune deficiencies secondary to aplasia or hypoplasia of the thymus, and hypocalcemia due to small or absent parathyroid glands. We now recognize that the phenotypic features seen in patients with DGS are much more variable and extensive than previously recognized (Botto et al., 2003; McDonald-McGinn et al., 1999; Ryan et al., 1997; Vantrappen et al., 1999; Table 45.2). The acknowledgment of similarities and phenotypic overlap with other disorders, including VCFS and CTAFS, as well as the association of all of these disorders with genetic defects in 22q11 has led to an expanded phenotype that includes palatal and speech abnormalities and cognitive, neurological, behavioral, and psychiatric difficulties.

IMMUNE DEFICIENCY

Most DGS patients have a mild to moderate immunodeficiency characterized by impaired production and function of T cells, with occasional secondary abnormalities in B-cell function (Barrett et al., 1981; Bastian et al., 1989; Junker and Driscoll, 1995; Mayumi et al., 1989; Schubert and Moss, 1992). The most obvious abnormality is the impaired production of T cells. T-cell counts are typically in the range of

Table 45.2 CLINICAL FEATURES OF THE 22Q11 DELETION SYNDROME

Fregnent
Conotruncal cardiac defects (49–83%)
Immune deficiency (80%)
Hypocalcemia (17–60%)
Palatal abnormalities (69–100%)
Cleft palate (9–11%)
Submucous cleft palate (5–16%)
Velopharyngeal insufficiency (27–32%)
Feeding and swallowing difficulties (30%)
Dysmorphic facies
Prominent nasal root
Narrow nasal alae with bulbous nasal tip
Hooded eyelids
Protuberant ears
Cup-shaped or thick, overfolded helices
Small mouth
Learning difficulties
Hearing loss (39%)
Renal anomalies (37%)
Less frequent
Thyroid disease
Microcephaly
Short stature
Slender hands and digits
Umbilical hernia
Inguinal hernia
Scoliosis
Skeletal anomalies
Hypospadias
Hematological abnormalities
Neuropsychiatric disorders

500 to 1,500 cells/mm^3 for the first months of life (Bastian et al., 1989; Junker and Driscoll, 1995). This defect in T-cell production is due to an anatomical thymus gland deficiency. Studies have demonstrated that most patients have either a very small anterior mediastinal thymus or small rests of thymic tissue scattered throughout the neck and submandibular areas (Bale and Sotelo-Avila, 1993; Lischner and Huff, 1975). This tissue consists of normal thymic components and is apparently sufficient to support normal T-cell maturation. Although decreased numbers of mature T cells are produced, the orderly sequence of maturation appears to be preserved.

The spectrum of immunodeficiency is broad. While the typical patient demonstrates impaired T-cell production in the first months of life, some DGS patients have normal or near-normal T-cell production, whereas a minority has almost

no detectable T-cell production. In general, the T-cell deficit improves during the first year of life, and 35 percent of DGS patients produce normal numbers of total T cells by the end of the first year of life (Jawad et al., 2001). There is some suggestion that DGS patients with a normal-appearing thymus are unlikely to have substantial persistent immunodeficiency (Bastian et al., 1989). In contrast, complete anatomical absence of a thymus does not preclude the ultimate development of normal immunological function.

Although all T-cell subsets are reduced, CD8 T cells are diminished the most. In early infancy the average CD8 count is approximately 300/mm^3 and the average CD4 count is approximately 850/mm^3 (Bastian et al., 1989; Junker and Driscoll, 1995). In an effort to understand whether peripheral blood T cells in patients with DGS develop aberrantly as a result of insufficient thymic epithelium, patient cells have been stained with markers specific for different stages of thymic maturation (Muller et al., 1989; Reinherz et al., 1981; Sirianni et al., 1983a, 1983b). On the basis of these studies, it appears that T cells use the usual developmental pathways; however, DGS patients are simply not able to produce as many T cells during the first months of life. It is not known why CD8 T cells are more profoundly affected than CD4 T cells.

T-cell function is usually grossly normal as measured by responses to T-cell mitogens (Barrett et al., 1981; Bastian et al., 1989; Junker and Driscoll, 1995; Muller et al., 1989). However, a small minority of patients have markedly diminished T-cell responses. In the past, this distinction has led to confusing nomenclature. Patients with minimal T-cell mitogen responses were said to have complete DGS and patients with normal or near-normal T-cell mitogen responses were said to have partial DGS. In fact, there is a spectrum of severity for all of the phenotypic features of this disorder. Some of the patients with markedly impaired T-cell function will not improve with time and will require either a bone marrow transplant or thymus transplant, whereas others will improve, although improvement becomes less likely after the first few months.

The consequences of T-cell deficiency in DGS patients are an increased susceptibility to infections, particularly viral infections, and an increased risk of autoimmune disease. Specifically, parainfluenza, adenovirus, and rotavirus have been shown to cause severe morbidity in DGS patients (Beard et al., 1980; Gilger et al., 1992; Tuvia et al., 1988; Wood et al., 1988). Most viral illnesses are more prolonged in DGS patients with compromised T-cell function. It is not known if decreased T-cell numbers are an independent risk factor for prolonged viral diseases in the absence of T-cell dysfunction. Two studies demonstrated that DGS patients have an impaired T-cell repertoire, suggesting that markedly diminished T-cell numbers do contribute to infectious morbidity (Pierdominici et al., 2000; Piliero et al., 2004). DGS patients with severely diminished T cells are also at risk from the same opportunistic infections as patients with severe combined immunodeficiency (SCID).

Juvenile rheumatoid arthritis, immune-mediated thrombocytopenia (ITP), and autoimmune hemolytic anemia occur with increased incidence in patients with DGS (Duke et al., 2000; Jawad et al., 2001; Pinchas-Hamiel et al., 1994). In addition, individual cases of autoimmune thyroiditis have been described, suggesting that there is a generalized increased frequency of autoimmune disease in DGS patients (Ham Pong et al., 1985). The delayed formation and maturation of T-cell regulatory functions might allow for the development and proliferation of autoreactive T cells (Sullivan et al., 2002).

The natural killer (NK) cell compartment often represents a large fraction of the total peripheral blood lymphocytes. In some cases, this reflects genuine expansion of this cell lineage. NK-cell function has been examined in DGS patients and found to correlate poorly with NK-cell number or T-cell studies (Muller et al., 1989).

In contrast to the quantitative deficiency of T cells, the B-cell compartment is typically normal or expanded. The B cells often represent a larger fraction of the total lymphocyte pool than in normal infants, and the absolute numbers of B cells may be expanded as well. Markedly increased B-cell numbers may correlate with poorer T-cell function (Jawad et al., 2001; Muller et al., 1989). This is thought to be due to aberrant regulation of B cells by the deficient T cells. There is evidence that there is defective T-cell suppression of B-cell immunoglobulin production in infants with DGS (Durandy et al., 1986), and it is not uncommon for infants with DGS to have hypergammaglobulinemia (Muller et al., 1988). On the other hand, hypogammaglobulinemia, IgA deficiency, and delayed acquisition of appropriate antitetanus and antidiphtheria antibody titers have been described in DGS patients (Junker and Driscoll, 1995; Mayumi et al., 1989; Smith et al., 1998). This is also thought to result from the relative deficiency of appropriate T-cell help for immunoglobulin production. A DGS kindred with persistently impaired T-cell production, normal T-cell function, and defective ability to form some antipolysaccharide antibodies has been described. The occurrence of similar humoral dysfunction in the same kindred suggests that modifier genes may play a significant role in the ultimate level of humoral function. In general, however, it is not possible to predict the level of immunological function of an affected child on the basis of previously diagnosed siblings or parents. Recognition that T-cell dysfunction could alter immunoglobulin production prompted a study of immunoglobulin diversity in DGS patients. The immunoglobulin repertoire was normal in a patient with severe T-cell deficits as well as in a patient with mild T-cell compromise. However, somatic mutation was found to be markedly deficient in the patient with significant T-cell dysfunction compared to the more mildly affected patient and normal controls (Haire et al., 1993).

OTHER FEATURES

Congenital Abnormalities

The most common cardiovascular malformations include interrupted aortic arch type B (14–15 percent), truncus arteriosus (7–9 percent), and tetralogy of Fallot (17–22 percent). Although neonatal hypocalcemia is not uncommon in DGS, recent studies suggest that patients are at risk for a spectrum

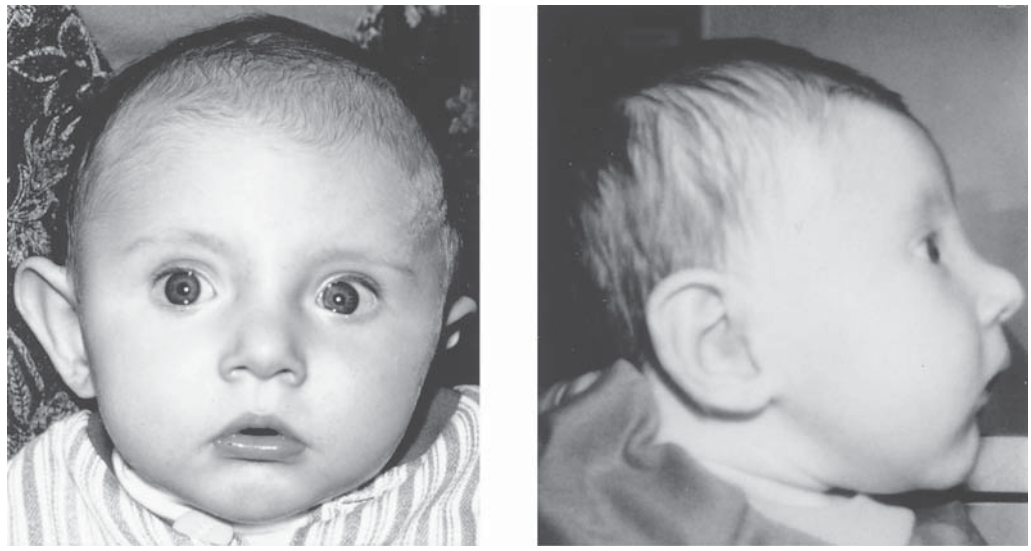

Figure 45.1 Frontal and side views of a 4-month-old boy with DGS and a 22q11.2 deletion. Note the prominent ears, bulbous nasal tip, mild micrognathia, and sloping forehead.

of parathyroid gland abnormalities including latent hypoparathyroidism, which may evolve to hypocalcemic hypoparathyroidism during adolescence or early adulthood (Cuneo et al., 1997). One study demonstrated that chromosome 22q11.2 deletions were seen in 10 of 14 neonates with hypocalcemia (Adachi et al., 1998).

Newborns and infants with DGS may have dysmorphic facial features such as widely spaced eyes (hypertelorism); low-set, prominent ears; and micrognathia. Later in childhood, adolescence, and adulthood their facial features evolve (Fig. 45.1). The characteristic facial features include a long face; a prominent nose with a bulbous nasal tip and narrow alar base; almond-shaped or narrow palpebral fissures; malar flattening; recessed chin; and malformed, prominent ears (Shprintzen et al., 1981). In addition, a spectrum of palatal abnormalities may be seen in DGS, such as cleft palate, submucous cleft palate, velopharyngeal insufficiency (VPI), bifid uvula, and cleft lip with or without cleft palate. Feeding difficulties and gastroesophageal reflux are common and can require tube feeding or a gastrostomy tube (Eicher et al., 2000). Renal anomalies such as single kidney, multicystic dysplastic kidney, horseshoe kidney, and duplicated collecting system occur in approximately one third of DGS patients. Hearing loss in one or both ears is not uncommon. Other features seen less frequently include microcephaly, short stature, hypospadias, ocular abnormalities, inguinal and umbilical hernias, hematological abnormalities, and skeletal anomalies (McDonald-McGinn et al., 1999; Ryan et al., 1997).

Neurocognitive, Behavioral, and Psychiatric Abnormalities

Structural malformations of the central nervous system such as neural tube defects, a small cerebellar vermis or posterior fossa, and polymicrogyria have been described, but it is unclear if these changes correlate with the developmental, cognitive or behavioral abnormalities observed in DGS patients (Nickel et al., 1994; Mitnick et al., 1994).

Prospective evaluations of children with DGS have enabled us to understand the range of cognitive and neuropsychological problems they are at risk to develop. In children of preschool age, developmental delay, motor delays, mild hypotonia, and language and speech delays, especially for expressive language, are common. Behavioral problems observed include attention-deficit/hyperactivity disorder (ADHD), inhibition, and shyness (Gerdes et al., 1999; Goldring-Kushner et al., 1985; Swillen et al., 1997). Psychoeducational and neurodevelopmental studies of children with the 22q11 deletion demonstrated full-scale IQ scores in the normal to moderately retarded range, with the majority falling in the borderline range (IQ 70–84). In early childhood, nonverbal learning difficulties are not uncommon, but there were no significant differences by adolescence. Math skills are in general lower than reading and spelling (Anthshel et al., 2010; Moss et al., 1999). Language difficulties persisted in approximately 50 percent of the children of school age (Solot et al., 2000). Adults demonstrate deficiencies in social judgment, motor skills, verbal learning, and executive function (Chow et al., 2006).

Recent studies also suggest an increased frequency of behavioral and psychiatric disorders. In children, ADHD, specific phobias, and anxiety are common (Anthshel et al., 2010; Niklasson et al., 2009), and autism spectrum disorders have been reported (Vorstmann et al., 2006). Psychiatric disorders, including generalized anxiety disorder and schizophrenia, have been diagnosed in about 60 percent of adults with the 22q11 deletion. The risk of developing schizophrenia or schizoaffective disorder is about 20 times the population risk (Fung et al., 2010). Prospective studies will provide greater insight into the risk factors for psychiatric disorders and may identify genetic variants in the region, which may be predictive (Philip and Bassett, 2011).

OTHER DISORDERS ASSOCIATED WITH THE 22Q1 1 DELETION

In addition to the overlap between DGS and VCFS, there are striking similarities between patients with DGS and those with CTAFS, a disorder predominantly described among the Japanese. CTAFS is characterized by conotruncal cardiac defects in association with a characteristic facial appearance. The facial features include ocular hypertelorism, lateral displacement of the inner canthi, flat nasal bridge, small mouth, narrow palpebral fissures, bloated eyelids, and malformed ears (Kinouchi et al., 1976). Some patients have a history of neonatal tetany, absent or hypoplastic thymus, and partial depression of T-cell–mediated immune responses. The finding of 22q11 deletions in CTAFS patients confirmed an earlier suggestion that CTAFS, VCFS, and DGS are the same entity (Burn et al., 1993; Matsuoka et al., 1994).

Recent cytogenetic and molecular studies of patients diagnosed with Opitz/GBBB syndrome suggest that there are two distinct genetic etiologies: chromosome 22q11 and a second on the X chromosome (McDonald-McGinn et al., 1995; Robin et al., 1995a). Opitz/GBBB syndrome (MIM 145410) is characterized by hypospadias, laryngotracheal anomalies, and hypertelorism (Opitz et al., 1965). Patients may have other features, including cleft lip and palate, cardiac defects, umbilical and inguinal hernias, cryptorchidism, imperforate anus, and facial dysmorphia such as telecanthus, prominent nasal bridge, or depressed nasal root with anteverted nares.

DiGeorge syndrome features have been observed in several other disorders, including CHARGE association (coloboma, heart defects, atresia of the choanae, retarded growth, genital hypoplasia, ear anomalies), Cayler cardiofacial syndrome, Kallman syndrome, and Noonan syndrome (Bawle et al., 1998; Lammer and Opitz, 1986; Robin et al., 1995b; Wilson et al., 1993a). However, the 22q11 deletion has not been found to be causally related to these disorders in a significant number of cases.

PHENOTYPIC VARIABILITY OF DGS

The variability in DGS arises from two issues. Patients with DGS can have diverse etiologies, such as diabetic mothers, the 22q11 deletion, or other genetic causes. In addition, patients who carry a common deletion with identical endpoints can have very diverse clinical features.

There is a wide range of phenotypic variability associated with the 22q11 deletion. While some individuals present with classic findings of DGS, others have relatively subtle features such as minor dysmorphic facial features or mild cognitive impairment. Prospective studies of newborns and children with conotruncal defects that were initially believed to be isolated indicate that those patients who have the 22q11 deletion often eventually manifest other features of the syndrome, such as mild facial dysmorphism, velopharyngeal insufficiency, and learning difficulties (McDonald-McGinn et al., 1997). Such studies suggest that all deletion-positive patients should receive comprehensive medical evaluations and follow-up as well as neuropsychological and educational assessments (Bassett et al., 2011; Oskarsdottir et al., 2005).

CYTOGENETIC AND MOLECULAR STUDIES

Cytogenetic studies of patients with DGS provided the initial evidence linking chromosome 22 to DGS (reviewed in Greenberg et al., 1988). Early reports of DGS patients with unbalanced translocations resulting in the loss of 22pter to q11 and two patients with interstitial deletions of 22q11 suggested that this region of chromosome 22 was important in the etiology of DGS. Molecular studies demonstrated that apparently cytogenetically normal DGS patients had submicroscopic deletions within 22q11 and defined the DiGeorge critical region (DGCR) (Driscoll et al., 1992a).

The presence of features common to DGS in patients diagnosed with VCFS prompted investigators to study VCFS patients for evidence of a 22q11 deletion (Fig. 45.2).

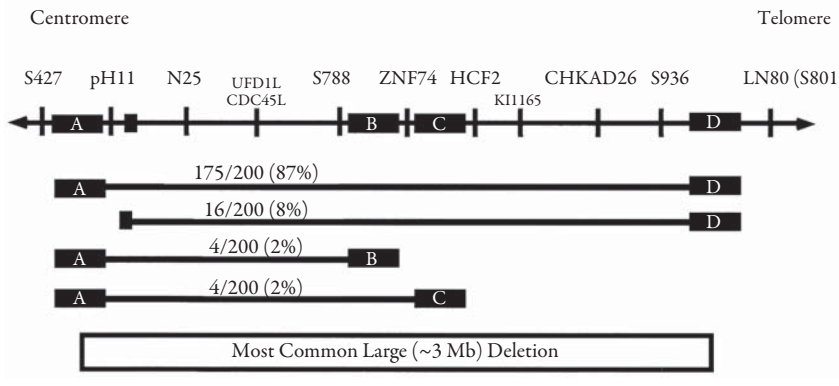

Figure 45.2 DiGeorge chromosomal region illustrating the size and location of the 22q11. 2 deletions in 200 patients. Deletion size and boundaries (A–D) were determined by fluorescence in situ hybridization (FISH), using as probes cosmids containing the genes and markers listed on the map of 22q11.2. The duplicated blocks of sequence or low copy repeats, designated A, B, C, D, coincide with observed deletion endpoints in patients. The extent and frequency of the four recurrent deletions are shown as lines below the map. Eighty-seven percent of DGS patients have a 3 Mb deletion extending from A to D. (Modified figure courtesy of B. S. Emanuel.)

Cytogenetic studies using high-resolution banding techniques detected interstitial deletions (del 22q11.21–q11.23) in 20 percent of VCFS patients (Driscoll et al., 1992b). Molecular studies with chromosome 22 probes previously shown to be deleted in patients with DGS demonstrated that the majority of individuals with VCFS have similar deletions of 22q11 (Driscoll et al., 1992b, 1993; Kelley et al., 1993). These studies coincided with the increasing utilization of fluorescence in situ hybridization (FISH) for the detection of microdeletions and subtle translocations. FISH of metaphase chromosomes by use of DNA probes from the DGCR led to a dramatic increase in the detection of 22q11 deletions in patients with features of DGS, VCFS, CTAFS, and conotruncal cardiac malformations (Color Plates 45.I and 45.II). The majority of DGS patients have a 3 Mb deletion. More recently, newer technology such as PCR-based multiplex ligation dependent probe amplification (MLPA) and array comparative genomic hybridization (CGH) have been used to detect smaller deletions nested within the 3 Mb deletion and atypical distal deletions in about 10 percent of patients with features of DGS and VCFS (Ben-Shachar et al., 2008; Busse et al., 2011; Jalali et al., 2008).

MECHANISM OF THE 22Q11 DELETION

Significant progress has been made in our understanding of the mechanism of the 22q11.2 deletion. Numerous DGS-associated translocations within the commonly deleted region and identification of unstable DNA sequences near the recurrent constitutional t(11; 22) translocation breakpoints suggest that 22q11.2 is an unstable region (Kurahashi et al., 2000). The region contains low copy number repeats (LCRs), which lead to nonallelic homologous recombination due to a misalignment during meiosis that results in deletions and duplications (Saitta et al., 2004; Shaikh et al., 2000, 2001). Analysis of the sequence of these LCRs indicates that they are large, highly homologous, and complex.

GENE IDENTIFICATION

At least 40 genes have been identified within the 3 Mb commonly deleted region of 22q11.2. This entire gene-rich region of chromosome 22 has been sequenced and is now available in the public domain (Dunham et al., 1999). Animal and human studies suggest that *TBX1*, a T-box transcription factor, is the responsible gene; however, there are several genes in the commonly deleted region that may contribute to the variable phenotypic features in DGS. They are described in this section.

The human *TBX1* gene was identified in the commonly deleted region and is deleted in the majority of DGS patients (Chieffo et al., 1997). *TBX1* is a member of a conserved family of genes that share a "T-box" DNA-binding domain and function as transcription factors involved in the regulation of developmental processes. Animal models strongly suggest a dominant role for TBX1 protein in the DGS phenotype (see "Animal Models") and have provided insight into the genetic pathway for DGS. It appears that Tbx1 is part of a regulatory pathway including non-chromosome 22 genes encoding Six1, Eya1, and Fgf8 that controls morphogenesis of the mammalian heart and face (Guo et al., 2011). The demonstration of point mutations in TBX1 leading to the full phenotype of DGS argues strongly for this being the major contributor to the phenotype (Yagi et al., 2003).

Several additional genes described in the common DGS deleted region may also contribute to the phenotype, given their expression patterns and potential role in cardiac and neural crest cell migration and differentiation. In 1995, *DGCR2/LAN/IDD*, a gene expressed in a variety of human adult and fetal tissues, was identified by several investigators (Budarf et al., 1995a; Demczuk et al., 1995; Wadey et al., 1995). This gene encodes a potential adhesion receptor that might mediate specific adhesive interactions between cells resulting in abnormal migration or interaction of the neural crest cells with the branchial arches.

The novel gene *DGCR6* is located in the proximal DGCR and shares homology with the *Drosophilia melanogaster* gonadal protein (gdl) and laminin g-1 (*LAMC1*) chain (Demczuk et al., 1996). Although *DGCR6* is present in all adult tissues except placenta, it appears to be most abundant in heart and skeletal muscle. Because of its homology with laminin, which, through its interactions with a receptor, plays a role in cell attachment, migration, and tissue organization during embryonic development, it is possible that *DGCR6* may play a role in neural crest cell migration. A goosecoid-like homeobox gene, *GSCL* or *GSC2*, has also been identified within the DGS region (Funke et al., 1997; Gottlieb et al., 1997). *GSCL* is expressed during early human development in the derivatives of the cephalic neural crest cells.

Catechol-*O*-methyltransferase encoded by the gene *COMT*, also within the commonly deleted region (Grossman et al., 1992), is important in the metabolism of catecholamines such as noradrenaline, adrenaline, and dopamine. A polymorphism has been identified, *COMT* 158^{met}, that results in decreased enzyme activity, and several studies have looked for an association with cognitive function and psychiatric disorders, with contradictory results (reviewed in Philip and Bassett, 2011). Another gene in the region, *PRODH*, encoding proline dehydrogenase, catalyzes the conversion of proline to pyrroline-5-carboxylate and has been implicated in the etiology of schizophrenia and schizoaffective disorders, but the role in patients with DGS remains to be determined (Philip and Bassett, 2011; Prasad et al., 2008).

Glycoprotein Ibβ, encoded by the gene *GP1BB* in 22q11.2, is a component of the major platelet receptor for von Willebrand factor (Budarf et al., 1995b). Defects in this receptor result in a rare autosomal recessive bleeding disorder, Bernard-Soulier syndrome (BSS), which is characterized by excessive bleeding, thrombocytopenia, and very large platelets. A single patient with features of BSS and VCFS with a 22q11 deletion has been described (Budarf et al., 1995b). Haploinsufficiency for this region of chromosome 22 unmasked a mutation in the promotor region of the remaining *GP1BB* allele, resulting in manifestations of BSS (Ludlow et al., 1996). Haplosufficiency

for *GPIBB* is likely to be responsible for the mild thrombocytopenia seen commonly in patients with deletions of 22q11.2 (Lawrence et al., 2003).

ANIMAL MODELS

The creation of mouse models has enhanced our understanding of the molecular basis of DGS. Initially, maps and sequence of the region of mouse chromosome 16 that is homologous to the DGS chromosomal region on 22q11 were constructed (Lund et al., 1999; Puech et al., 1997; Sutherland et al., 1998). Targeted mutations of single genes and deletions of the homologous region of 16 have been developed to elucidate the role of individual genes or sets of genes in DGS. Most of the initial attempts failed to recapitulate the DGS phenotype. Eventually, using Cre-loxP chromosome engineering, Lindsay et al. (1999) created a mouse with cardiac defects similar to those seen in DGS. The mice had a 1.2 Mb deletion, designated *Df1*, of chromosome 16 containing at least 20 genes that are most often deleted in DGS patients. Most of the mice heterozygotes for this deletion were viable; 18 to 26 percent had abnormalities of the fourth aortic arch arteries but no other manifestations of DGS. Subsequently, through chromosome engineering and P1 artificial chromosome transgenesis, these investigators identified *Tbx1* as the gene in the *Df1* mouse responsible for the cardiac defects (Lindsay et al., 2001). A second group also used the Cre-loxP strategy to generate a slightly larger 1.5 Mb deletion in mouse chromosome 16 that results in conotruncal cardiac anomalies (interrupted aortic arch type B, vascular ring, right aortic arch) and absent parathyroid glands (Mersher et al., 2001). They also used a rescue transgenesis technique and concluded that *Tbx1* was the critical gene for cardiac defects in mice with both deletions. In addition to the cardiac defects, the transgenic mice had a smaller-than-normal thymus, occasionally with small ectopic thymic lobules, along with a reduced number of CD4 and CD8 cells; however, the parathyroids were normal. Similar to humans, the cardiac defects in heterozygous *Df1* mice are not fully penetrant. This mouse model has provided insight into the pathogenesis of the cardiac defects and the basis for reduced penetrance. The aortic arch defects appear to correlate with a delay and occasional failure of the differentiation of vascular smooth muscle from neural crest cell mesenchyme. Migration of the neural crest into the fourth arch and initial formation of the fourth pharyngeal arch arteries proceeds normally in the *Df1* mice, but the affected vessels are impaired in their growth and do not acquire smooth muscle (Lindsay and Baldini, 2001). Many of the heterozygous *Df1* embryos were capable of overcoming this vascular defect and developed normally. These studies suggest that, contrary to previous beliefs, the cardiac defects in DGS are secondary to delayed growth and responsiveness to developmental signals for remodeling of the vasculature. This finding illustrates how the animal model has altered our understanding of DGS as a developmental disorder.

At the same time, Jerome and Papaioannou (2001) reported on the production of a null mutation in mouse *Tbx1*. The heterozygotes were viable, but in 19 to 45 percent of them, depending on the parental strain of mouse, the fourth aortic arches were absent or reduced in thickness. There were no differences in the weight of the thymus, absolute number of thymocytes, or cell-surface markers CD3, CD4, and CD8 in *Tbx1* heterozygote versus wild-type mice; however, the *Tbx1* homozygous mutants were nonviable. Embryos that were recovered displayed a range of phenotypic findings similar to DGS, including short neck, abnormal facial structure, low-set or abnormally folded ears, single cardiac outflow tract, right aortic arch or a double aortic arch, absent thymus, and absent parathyroids. Other investigators have confirmed the cardiac defects in *Tbx1* mutants (Lindsay et al., 2001; Merscher et al., 2001). In the mouse, a *Tbx1* mutation is sufficient to produce a DGS phenotype. The anomalies in the *Tbx1* knockout mouse model correlate with the expression pattern of *Tbx1* in the embryonic mouse head mesenchyme, pharyngeal arches and pouches, and the otic vesicle. This study provides further evidence that in humans the *TBX1* gene is a key gene in DGS (Jerome and Papaioannou, 2001).

The *Ckrol* mouse mutant developed by Guris et al. (2001) suggests that other genes may contribute to the classical features of DGS. *Crkol* is highly expressed in the branchial arches during mouse embryogenesis and maps to mouse chromosome 16 outside the *Df1* deletion. Homozygous mice with a null mutation in *Crkol* have cardiac defects, including double-outlet right ventricle and ventriculoseptal defect with an overriding aorta due to abnormal alignment of the outflow tract. Approximately 40 percent of the mutant embryos also had an abnormal thymus, parathyroid, and thyroid glands, and mild skeletal anomalies of the head. The similarities to DGS suggest that this gene may contribute to the development of at least some of the clinical features.

CLINICAL AND LABORATORY DIAGNOSIS

DGS patients with the 22q11 deletion are ascertained through a variety of medical specialty clinics on the basis of presenting features. Recognition of the 22q11 deletion syndrome is important so that the medical community and schools can meet these patients' diverse needs. Furthermore, identification of a 22q11 deletion enables the clinician to provide the affected patient and his or her family with a more accurate assessment of their recurrence risk. Although deletions of 22q11 may be detected in 20 to 25 percent of DGS patients by means of high-resolution cytogenetic analysis, FISH of metaphase chromosomes or array CGH is more efficient (Desmaze et al., 1993; Driscoll et al., 1993; Wilson et al., 1992). Many commercial and hospital-based laboratories are using FISH with the probes from the DGCR for the clinical and prenatal detection of the 22q11 deletion (Color Plate 33.I). However, array CGH is recommended when a diagnosis of DGS is suspected and FISH fails to identify a 22q11 deletion (Busse et al., 2011). Gene sequencing of *TBX1* may be considered in patients strongly suspected of having DGS in whom FISH and array CGH is normal.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

The majority of the 22q11 deletions are sporadic events, occurring only in the affected individual. The incidence of familial cases of DGS is approximately 10 percent (McDonald-McGinn et al., 2001). Hence, it is important to closely examine the parents of an affected child for subtle features, such as mild facial dysmorphia, hypernasal speech, and history of poor school performance, that are suggestive of a 22q11 deletion. Deletion testing of the parents is recommended. A parent or proband with a 22q11 deletion has a 50 percent chance of transmitting the deletion-bearing chromosome to his or her offspring. Parents with a 22q11 deletion benefit from preconception and antenatal genetic counseling to review their risk of having affected offspring and to discuss their reproductive options, including prenatal testing and preimplantation genetic diagnosis (Driscoll, 2001; Iwarsson et al., 1998).

Antenatal detection of the 22q11 deletion by FISH has been successfully performed on cultured amniocytes and chorionic villi obtained from at-risk pregnancies (Driscoll, 2001; Driscoll et al., 1993). Pregnancies considered at high risk include those of deletion-positive parents or parents with mosaicism for the 22q11 deletion, and those in which ultrasound or echocardiography demonstrates a fetal conotruncal cardiac malformation. Although the recurrence risk for normal parents with a previous affected child is low, parents often request prenatal testing for the deletion to exclude the small possibility of germline mosaicism. Although testing for the deletion is highly accurate, it is impossible to predict the phenotype. The size and extent of the deletion have not shown correlation with the penetrance and severity of features of DGS (Lindsay et al., 1995). There is significant intrafamilial variability, and the parental phenotype is not a reliable predictor of outcome for the fetus (McDonald-McGinn et al., 2001). Fetal imaging techniques such as ultrasonography and fetal echocardiography are recommended to evaluate the fetus for the presence of a cleft palate, renal anomaly, or cardiac defect. Subtle congenital anomalies such as a submucous cleft palate and dysmorphic features cannot be appreciated by ultrasonography (Driscoll, 2001).

At the present time, it remains difficult to counsel families with children with DGS unless an etiology has been identified. For now, fetal sonography and echocardiography are the only available prenatal tests that are helpful when evaluating at-risk pregnancies that are not causally related to an abnormality of 22q11 or other recognizable chromosomal abnormality.

MANAGEMENT AND TREATMENT OF THE IMMUNODEFICIENCY

The clinical management of infants and children with 22q11 deletion syndrome depends on their degree of immunocompromise. Most infants with the classic features of DGS are diagnosed in the first weeks of life through their characteristic cardiac anomalies and hypocalcemia, whereas older children may not be recognized until they develop speech and/or learning problems. As soon as the diagnosis of the 22q11.2 deletion syndrome or DGS is established, the individual should have an initial immunological evaluation. It is important to specifically evaluate immunological function regardless of the severity of the other phenotypic features (Sullivan et al., 1998). Depending on the age of the child and the level of concern, this could include T- and B-cell subset analyses, T-cell mitogen and antigen responses, immunoglobulin production, and tests of specific antibody formation.

Newborns should be evaluated for T-cell production and function by analyzing lymphocyte subsets at a minimum. Very young infants with markedly diminished T-cell numbers and function may be followed for a period of time to determine whether the immunodeficiency persists (Sullivan et al., 1999). During this time, they are at risk for opportunistic infections and should receive prophylaxis for *Pneumocystis jiroveci* pneumonia (PCP), reverse isolation, irradiated blood products, and careful monitoring. They should not receive live viral immunizations. Persistence of a SCID-like phenotype requires either a bone marrow or thymus transplant (Markert et al., 1999, 2003) to restore immunological function (Goldsobel et al., 1987; Markert et al., 2007). Because both interventions carry significant risk and the immunodeficiency of DGS is developmentally expressed, it seems wise to delay intervention for 1 to 2 months when possible.

Most infants with 22q11 deletion will have a mild immunodeficiency with an excellent long-term prognosis. T-cell production is usually moderately impaired and T-cell mitogen responses are often normal or near normal. Strategies such as PCP prophylaxis and use of killed vaccines for the patient and family contacts may be considered for infants with very low T-cell counts. Most infants will develop normal or near-normal T-cell counts over time. However, the first year of life represents a vulnerable time during which they require careful monitoring. Although there are limited data to support specific management strategies, a reasonable approach to minimize risk to the affected infant is to withhold live viral vaccines until T-cell function is normal and the child has demonstrated an ability to form functional antibodies. Using the criteria of a CD8 count of at least 250/mm^3 and normal antibody responses to diphtheria and tetanus, we and others have not observed any serious side effects with OPV (oral polio vaccine), MMR (measles, mumps, and rubella vaccine), or varicella vaccine administration (Moylett et al., 2004; Perez et al., 2003). The recent widespread use of inactivated poliovirus vaccine (IPV) has limited early infant exposure to live viral vaccines. To date there have not been serious adverse events with the live rotavirus vaccine in patients with 22q11 deletion syndrome, though vaccine strain diarrhea has been reported in infants with SCID. PCP prophylaxis is required only for patients with extremely low T-cell counts. Sequential follow-up studies are important because the pace of immunological recovery is very unpredictable. Furthermore, there are no laboratory studies or phenotypic markers that predict ultimate immunological outcome. A typical strategy would be to measure lymphocyte subsets and mitogen responses at the time of diagnosis. The T-cell proliferative responses track very well with the T-cell counts; therefore, for many infants the measurement of T-cell counts is sufficient. If the mitogen responses are normal and the T-cell

Table 45.3 SUGGESTED IMMUNOLOGICAL EVALUATIONS OF NEWBORNS AND INFANTS WITH DGS

IMMUNOLOGIC EVALUATIONS	TIME OF DIAGNOSIS	AGE 4 MONTHS	AGE 8 MONTHS	AGE 12 MONTHS
Lymphocyte subsets	X	X	X	X*
Mitogen responses	X*			
Antigen responses				X*
Humoral function				X*

*Requires periodic re-evaluation if abnormal.

production is mildly to moderately impaired, the lymphocyte subsets should be repeated every 4 to 12 months until they have normalized or stabilized (Table 45.3). At 8 to 12 months of age, an analysis of humoral function should be performed. This can be quite helpful for determining the risk/benefit analysis for live viral vaccines. After a year of age, mitogen and antigen responses should be followed sequentially if abnormal on the first evaluation. More severely affected infants require more careful follow-up, while less severely affected infants may require somewhat less frequent follow-up.

Patients with all the elements of DGS at birth typically have a significant immune deficiency by definition. Early management of DGS patients often revolves around their cardiac anomaly, and it may be difficult to adequately assess the patient's immunological function. Blood transfusions and hemodynamic instability may require that the evaluation be limited or delayed. It is prudent to consider the patient as severely immunodeficient until such time as appropriate studies can be done. Because several cases of graft-versus-host disease due to blood transfusions have been recorded in DGS, and because early infancy is the most vulnerable time for DGS infants, only irradiated and cytomegalovirus (CMV)-negative blood products should be given (Brouard et al., 1985; Muller et al., 1989; Washington et al., 1993, 1994; Wintergerst et al., 1989).

In older children with DGS or 22q11 deletion syndrome, it is possible to examine immunological function thoroughly at the first evaluation. Patients with normal or near-normal T-cell production, normal T-cell mitogen and antigen responses, and a demonstrated ability to form functional antibody do not require special treatment or restrictions. Some patients have persistent mild T-cell abnormalities. These patients require individualized management, depending on the degree of T-cell compromise and the extent to which they develop significant infections. These patients also require follow-up studies because the immunological changes can be unpredictable. Most immunological improvement occurs in the first year of life, but additional T-cell function and improved T-cell production can occur later. Furthermore, thymic involution occurs as they get older, and they can have a decline in peripheral blood T-cell counts (Piliero et al., 2004).

Despite laboratory evidence of normal immunological function, many patients have recurrent upper respiratory infections due to abnormal palatal anatomy. These patients benefit from the same surgical interventions as other children, and prophylactic antibiotics as adjunct therapy may be considered. Allergies occur more frequently in 22q11 deletion syndrome than in the general population, and this too can contribute to the recurrent infections (Staple et al., 2005).

Since the majority of DGS patients will ultimately have normal or near-normal immunological function, most research efforts have been directed at improved management of the severely affected infants. Bone marrow transplantation (non-T-depleted) from an HLA-matched sibling donor has been efficacious (Borzy et al., 1989; Goldsobel et al., 1987). It may provide sufficient numbers of mature T cells to allow adequate T-cell function even in the absence of thymic tissue. Early therapeutic efforts were directed at replacing the missing thymic tissue and used fetal thymic grafts (Thong et al., 1978; Touranine, 1979). Although a minority of these grafts succeeded, the limitations imposed by the use of fetal tissue have compromised their practical application. Recently, success has been reported using discarded postnatal thymic tissue obtained during cardiac surgery grafts (Markert et al., 1995, 1999, 2003, 2004, 2007). Four months after placement of a thymic graft, mature functional host T cells were identified in peripheral blood. Antigen-specific T-cell responses developed and B-cell function normalized. This management strategy appears to offer many advantages over fetal thymus or bone marrow transplantation for severely affected DGS patients, although involution of the thymic graft may be a long-term issue.

A specific management issue for patients with DGS and no naïve T cells is the development of an Omenn-like picture (Markert et al., 2004). This is due to escape clones of T cells infiltrating skin and other organs and is just as dangerous as Omenn syndrome associated with SCID. The patients require strong immune suppression prior to definitive therapy. More important than the complex management issues that arise is the diagnostic conundrum. These patients have normal or even high T-cell counts in the peripheral blood. This can cause diagnostic confusion. A close examination of the phenotype of the T cells will reveal that they are all of a memory phenotype (CD45RO). This relatively subtle finding can be a very important diagnostic clue.

FUTURE CHALLENGES

DGS is characterized by a wide range of congenital malformations, facial dysmorphia, endocrine and immune disorders,

cognitive impairment, and neurological and psychiatric abnormalities. Cytogenetic and molecular studies have contributed greatly to our understanding of the genetic basis of this disorder. These studies have shown that deletions of chromosomal region 22q11 are the leading cause of DGS. More recently, *TBX1* was identified as a major contributor to the phenotype. Other genes within or outside the critical region may mold the phenotypic manifestations. The broad availability of genome-wide studies of single nucleotide polymorphisms may allow risk stratification of patients to be more proactive in management.

Prospective studies of DGS patients with 22q11 deletions continue to further delineate the range and severity of the medical, cognitive, and neuropsychological problems for which these patients are at risk. The wide range of phenotypic variability remains poorly understood. Neither the size of the deletion nor the parental origin, a factor in some genetic syndromes because of differences in chromosomal imprinting, appears to explain the penetrance or severity of the phenotype. Murine studies suggest that genetic background is an important influence on the phenotype. Therefore, we hypothesize that the severity and phenotypic variability in DGS may be due to modifier genes. Genetic studies of potential modifying genes in both humans and animal models may further our understanding of this complex disorder and eventually lead to more accurate prediction of the outcome and need for specific therapeutics and intervention. In the meantime, we urge clinicians to pursue comprehensive evaluations by a team of medical professionals in genetics, cardiology, immunology, endocrinology, plastic surgery, speech and hearing, and developmental pediatrics who have the expertise in caring for individuals with DGS.

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46.

INTRODUCTION TO DISORDERS ASSOCIATED WITH DNA REPAIR AND METHYLATION DEFECTS

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Our genetic material represents a thread of double helical DNA composed of around 3×10^9 base pairs. To compress this material into the small dimensions of a cell, the DNA is highly compacted by sophisticated and defined mechanisms, which collectively represent the higher-order chromatin structure. However, during the process of development, cells undergo defined DNA transactions that necessitate transient or limited relaxation of the compacted DNA structure. The development of the immune response is one such process. Our cellular DNA is also subjected to constant damage, which can arise from both endogenously generated and exogenous DNA-damaging agents. Proteins, particularly histones, which coat the DNA, provide one mechanism to afford protection from such damaging agents, but it is far from an efficient barrier, and DNA damage still ensues. The cell's major strategy for protection lies in an array of DNA damage-response mechanisms that repair damaged DNA or activate processes that optimize the opportunity for repair. Failing all that, cells have processes that eliminate damaged cells from the cycling population. However, the operation and optimal functionality of these damage-response mechanisms often necessitates the decondensation or relaxation of DNA from its compacted state. It is, therefore, not unsurprising that there is an interesting *ménage à trois* between the development of the immune response, the DNA damage response, and processes that regulate higher-order chromatin structure.

The major aim of the DNA damage-response mechanisms and, indeed, a role for higher-order chromatin structure is to help maintain genomic stability, since genomic instability can lead to cell death or, as significantly, enhance steps in the etiology of carcinogenesis. The development of the cognate immune response has the opposite aim—the generation of genetic diversity to create a repertoire of distinct T and B cells that are able to recognize the vast numbers of antigens to which we are constantly exposed. To achieve this, cells have subverted the DNA damage-response mechanism to help generate genetic diversity rather than maintain stability (Fig. 46.1). In these processes, defined lesions and/or breaks are created in DNA during immune development, which are then rejoined or repaired in a manner creating DNA sequence changes—quite the opposite endpoint that aims to be achieved by the damage-response mechanisms.

In this chapter, we will overview the damage-response mechanisms of relevance to immune development, consider how they have been supplanted to create diversity during immune development, and consider recent advances in our understanding of the impact of chromatin structure on both the response to DNA damage and immune development. Finally, we will consider how the DNA damage-response processes and chromatin structure affect other aspects of development, which is relevant to considering the basis underlying the additional clinical features that are characteristic of this class of immunocompromised patients and serve as useful differential diagnostic indicators.

DNA DAMAGE RESPONSES RELEVANT TO DEVELOPMENT OF THE IMMUNE RESPONSE

RANGE OF DNA LESIONS ENCOUNTERED BY A CELL

Reactive oxygen species (ROS) are a major contributor to DNA damage and by introducing purine and pyrimidine base and/or deoxyribose sugar damage can lead to both DNA single-strand breaks (SSBs) and DNA double-strand breaks (DSBs). Base modifications, such as methylation, can be

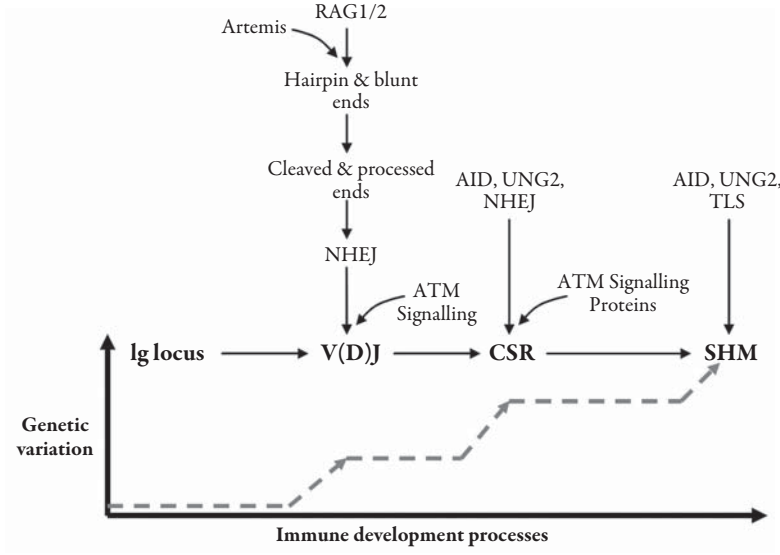


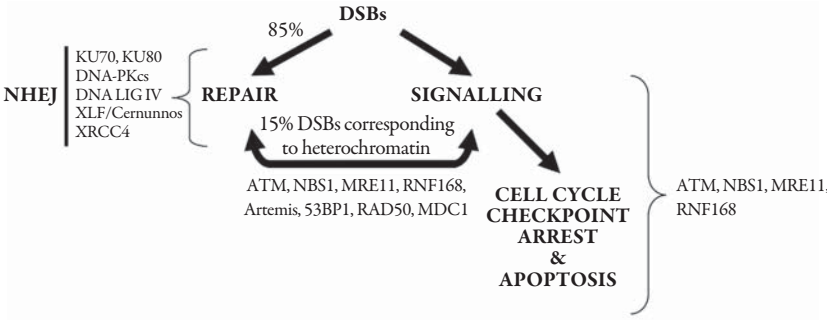
Figure 46.1 *How the immune response exploits DNA damage-response processes.* DNA damage-response processes are used by somatic cells to maintain genomic stability. During development of the immune response, these processes are subverted to generate genetic diversity. This figure highlights the stages of immune development that utilize DNA damage-response proteins. RAG1/2, recombination activating gene proteins 1 and 2; NHEJ, DNA nonhomologous end-joining; AID, activation-induced cytidine deaminase; UNG2, uracil-N-glycosylase 2; TLS, translesion synthesis.

induced by DNA alkylating agents present in our environment (e.g., methyl chloride) but can also arise in a programmed manner as a consequence of epigenetic gene programming during development. DNA bases can also undergo chemical changes such as spontaneous deamination and depurination. SSBs and DSBs can be introduced into DNA by topoisomerase I and II to relieve torsional stresses built up in the DNA double helix following transcription and replication, although these remain hidden, for the most part, as protein bridged lesions. DSBs are the most critical lethal lesion for a cell and can arise directly from DNA-damaging agents, most importantly from exposure to radiation, or via the processing of other lesions. As will be discussed in more detail below, the lesions of most significance in the context of immune development are DSBs, SSBs, and deamination events.

RESPONSES TO DNA DSBS

Despite the fact that a DNA DSB is a potentially devastating lesion, the programmed introduction of DSBs is a critical step during the development of the immune response, being a prerequisite for both V(D)J recombination and class-switch recombination (CSR) (Gellert, 2002; Mahaney et al., 2009; Manis et al., 2002). Since the inability to appropriately respond to DSBs enhances genomic instability, it is not surprising that certain genomic instability disorders display immunodeficiency (see Fig. 46.2 for an overview). To help set the stage for understanding the basis of this overlap, we provide below an overview of the DNA damage response (DDR) to DSBs.

The DDR encompasses pathways of DNA repair and damage-recognition signal transduction pathways. The DNA repair pathways are lesion or lesion-type specific, while the signal transduction pathways recognize one of two broad classes of lesions, a DNA DSB or a single-stranded region of DNA (Shiloh, 2001). DNA nonhomologous end-joining (NHEJ) represents the major DSB repair pathway, with homologous recombination (HR) repairing one-ended DSBs that arise at stalled replication forks as well as a subset of the DSBs that arise in G2 phase (Wyman et al., 2006). The kinase ataxia telangiectasia mutated protein (ATM) lies at the apex of the signal transduction pathway that responds to DSBs (Lavin 2008). Six core components of NHEJ have been identified that associate as two distinct complexes (Mahaney et al., 2009). The Ku protein, encompassing subunits Ku70 and Ku80, rapidly binds to DSBs and protects them from nucleolytic degradation. Once DNA bound, Ku recruits the DNA protein kinase catalytic subunit (DNA-PKcs), creating the DNA-PK complex and activating DNA-PK activity. The precise function of DNA-PK remains unclear, but increasing evidence suggests a role in promoting DNA end processing. DNA-PK also facilitates the recruitment of DNA ligase IV, XRCC4, and XLF/Cernunnos, which carry out the rejoining step of NHEJ. XRCC4 and DNA ligase IV interact very tightly and XRCC4 is required for the stabilization of DNA ligase IV (Grawunder et al., 1998). XLF/Cernunnos, in contrast, is a weaker binding partner of the DNA ligase IV/XRCC44 complex. In vitro it stimulates the ability of DNA ligase IV to rejoin incompatible DNA ends but has also been suggested to promote the in situ recharging of DNA ligase IV to effect the dual ligations required for DSB rejoining (Riballo et al., 2009; Tsai et al., 2007). In G1-phase cells, these core NHEJ proteins are required for the majority of DSB rejoining, and hence cells lacking any of these proteins display dramatic radiation sensitivity (O'Driscoll and Jeggo, 2006). Following exposure to X- or gamma-rays, two components to DSB rejoining can be distinguished: 85 percent of the DSBs are rejoined with fast kinetics, while approximately



Gene	Disorder
ATM	Ataxia telangiectasia (A-T)
NBS1	Nimegen breakage syndrome (NBS)
MRE11	A-T-like Disorder
RAD50	NBS-like disorder
RNF168	RIDDLE syndrome
DNA-PKcs	DNA-PKcs-dependent-SCID
ARTEMIS	Artemis-dependent-SCID
XLF/Cernunnos	XLF-Cernunnos-dependent SCID
DNA LIG IV	LIG4 syndrome

Figure 46.2 *Disorders conferring genomic instability and immunodeficiency: the point of impact of the defective protein in the DNA damage response.* DNA DSBs are an important lesion induced during immune development to create genetic diversity. Thus, there is a strong overlap between disorders affecting the damage response to DSBs and immune development. The DSB damage response encompasses pathways of DSB repair and a signal transduction response. The signaling response is required for the repair of a subset of DSBs as well as cell-cycle checkpoint arrest and apoptosis. The figure shows where key proteins function in the DNA damage response and identifies disorders caused by their loss.

15 percent are rejoined with markedly slower kinetics (Lobrich et al., 1995; Wu et al., 2008). Recent studies have shown that the slow component of DSB repair requires a number of additional proteins, including ATM and the nuclease, Artemis (Riballo et al., 2004). Further, these have been suggested to be DSBs that arise within or close to heterochromatic (HC) DNA (Goodarzi et al., 2008). The role of ATM in this aspect of DSB repair has been proposed to be the phosphorylation of Kap1 (Kruppel-associated box [KRAB]-associated protein), a heterochromatic building factor, thereby relaxing chromatin compaction to promote DSB repair within this more compacted DNA region (Ziv et al., 2006). The role of Artemis is unclear but potentially represents the removal of secondary structures or damaged lesions at the DNA ends.

ATM-dependent signaling represents an elegantly choreographed assembly of factors at the DSB (Panier and Durocher, 2009). Despite extensive studies on understanding the assembly process, its precise function is still unclear, although aiding the repair of DSBs located within the compacted heterochromatic regions represents one critical endpoint (Goodarzi et al., 2008). Additionally, ATM-dependent signaling activates cell-cycle checkpoint arrest, a process that enhances the opportunity for DSB repair by delaying progression through critical cell-cycle stages (Lavin, 2008). Cell-cycle checkpoint arrest can also function as an alternative to apoptosis to permanently remove damaged cells from the cycling population (Wahl et al., 1997). The Mre11/Rad50/Nbs1 (MRN) complex represents the initial DNA damage sensor that, via its binding to ATM, recruits ATM to the DSB end, a process analogous to the way in which Ku recruits DNA-PKcs to the DNA end (ATM and DNA-PKcs are related phosphoinositol 3-kinase-like kinases) (Falck et al., 2005; Mirzoeva and Petrini, 2003; Uziel et al., 2003). In undamaged cells, ATM exists as a dimer but undergoes monomerization and activation when recruited to the DSB (Bakkenist and Kastan, 2003). An early target of ATM kinase activity is the histone H2A variant, H2AX. H2AX represents around 1 to 10 percent of the H2A molecules, and phosphorylated H2AX, designated γH2AX, can extend large distances from the DNA end to generate defined foci, which can be readily visualized by immunofluorescence using specific antibodies (Paull et al., 2000). γH2AX serves to recruit MDC1, one of several mediator proteins responsible for the formation of DSB "repair foci." MDC1 recruitment aids the recruitment of two ubiquitin ligases, RNF8 and RNF168, which ubiquitinate histone H2A residues, which in turn serves to recruit 53BP1, another mediator protein (Panier and Durocher, 2009). BRCA1 is also recruited to the DSB in an MDC1-dependent manner and appears to contribute to H2A ubiquitination. Significantly, all these proteins are also required for the slow component of DSB repair in G1 phase while being dispensable for the fast rejoining process (unpublished observations).

DSB REJOINING IN THE CONTEXT OF THE IMMUNE RESPONSE

V(D)J recombination and CSR represent two critical processes in the development of the immune response that involve the programmed introduction of DSBs (Gellert, 2002; Mahaney et al., 2009; Manis et al., 2002). Following this, both processes subvert the NHEJ rejoining pathway to promote sequence changes at the rejoined DNA junctions. The first step in V(D)J recombination is the creation of a DSB by the combined action of the RAG1 and RAG2 endonucleases (Gellert, 2002;

Oettinger et al., 1990) (see also Chapter 13 for further details of this process). RAG1/2 cleavage creates a single strand nick followed by a trans-esterification reaction that generates a DSB with one blunt opposing a hairpin-sealed DNA end (Kim et al., 2000). In fact, the broken ends are associated in a distinct manner to a radiation-induced DSB with four DNA ends generated from two cleavage events being associated as a synaptonemal complex. The two hairpin DNA ends undergo cleavage and rejoining to generate coding junctions that form the coding sequences of the immunoglobulin or T-cell-receptor genes. The two blunt ends are rejoined to generate signal junctions that are dispensable for the immune response. The rejoining step of V(D)J recombination involves the core NHEJ proteins, and cell lines or mice lacking Ku, DNA-PKcs, XRCC4, DNA ligase IV, or XLF/Cernunnos are impaired in their ability to carry out V(D)J recombination. The hairpin cleavage reaction requires the nuclease, Artemis. Since hairpin cleavage is a prerequisite for rejoining of the ends generated during V(D)J recombination, Artemis is an essential V(D)J recombination component: in contrast, it is only required for the repair of a subset (~15 percent) of the radiation-induced DSBs (as discussed above).

To date, patients with mutations in DNA ligase IV, XLF/Cernunnos, Artemis, and DNA-PKcs have been identified (see Chapters 9 and 13 for detailed discussion of these patients). Such patients display severe combined immunodeficiency (SCID) or CID consistent with an inability or impaired ability to carry out V(D)J recombination. Cell lines from such patients display radiosensitivity and DSB repair defects, a feature exploited for their clinical diagnosis. Importantly, such patients are likely to display clinical radiosensitivity, an outcome that, for at least one undiagnosed patient, proved fatal (Riballo et al., 2001). Moreover, these patients can also display sensitivity to DNA-damaging agents that, directly or indirectly, lead to enhanced DSB formation. This aspect is critical in considering conditioning regimens for bone marrow transplantation for such patients (O'Driscoll and Jeggo, 2008a).

Although neither ATM nor the MRN complex is essential for V(D)J recombination and neither ataxia-telangiectasia (A-T) nor Nijmegen breakage syndrome (NBS) patients (who are defective in *ATM* and *NBS1*, respectively) display the SCID or CID phenotypes characteristic of patients with mutations in NHEJ proteins, aberrant V(D)J junctions in the form of translocations involving chromosomes 7 and 14 are a characteristic feature of both conditions (Hsieh et al., 1993) (see Chapters 47 and 48). Additional recent studies have also demonstrated aberrant V(D)J recombination products in lymphocytes from A-T and NBS patients or mouse models (Bredemeyer et al., 2008b; Callen et al., 2007; Helmink et al., 2009; Huang et al., 2007). There are several possible mechanisms by which ATM might influence V(D)J recombination. Firstly, rather than being critical for the rejoining step, ATM may regulate the fidelity of the process. Evidence for this is the observation that hybrid joint formation, representing the aberrant rejoining of coding and signal ends, occurs at an elevated frequency in ATM-deficient lymphocytes via a process that requires the NHEJ proteins (Bredemeyer et al., 2008b). Secondly, apoptosis and/or cell-cycle checkpoint arrest appears to be an important physiological step in T- and B-cell development, most likely serving to remove cells that have failed to undergo productive rearrangements or those that retain unrejoined DSBs. The persistence of cells with chromosome breaks in the absence of ATM supports a role for ATM in preventing the transmission of lymphocytes that have failed to complete V(D)J recombination (Callen et al., 2007). Indeed, DSB formation in lymphocytes activates a specific signaling program that is essential for normal lymphocyte development and is distinct from the normal DSB response in other cell types (Bredemeyer et al., 2008a).

CSR is a second process that also involves the generation of a DSB via a very distinct mechanism. This process and its link to the DDR will be discussed later in this chapter.

DNA DAMAGE RESPONSES TO SSBs AND BASE DAMAGE

Despite the high level of formation of base damage and SSBs, they are rarely lethal lesions, which is due primarily to the abundant, overlapping, and highly efficient pathways for their repair and the fact that they induce mutational changes rather than lethality if not repaired efficiently. Base excision repair (BER) represents the major DNA pathway that removes base damages. This process will not be reviewed in detail (see Caldecott, 2008, for an excellent overview). In brief, the first step of BER is recognition of a damaged base by a DNA glycosylase. DNA glycosylases are not entirely lesion specific but have strong lesion preference, and thus a battery of distinct glycosylases recognize distinct forms of base damages. The glycosylase removes the damaged base, leaving either an apurinic or apyrimidinic site or an SSB if the glycosylase has associated lyase activity. Apurinic-apyrimidinic (AP) endonuclease I (APE1; also known as APEX1) readily converts the abasic site into a SSB. The SSBs generated by either these two mechanisms differ, but both require further processing to create the 5′ phosphate and 3′ hydroxyl ends necessary for ligation. DNA polymerase β carries out any fill-in of bases necessary during BER. Finally, XRCC1 and DNA ligase III carry out the rejoining step of BER.

Most base damage is repaired prior to replication. However, occasionally such damages are encountered at the replication fork and cause replication fork stalling or even replication fork collapse. Homologous recombination (HR) represents one process to bypass and subsequently repair such lesions at the replication fork. Another process is translesion synthesis (TLS). In this process, the stalled replicative polymerase is switched to one of several specialized error-prone polymerases, which have reduced fidelity and can replicate past a damaged base or lesion, albeit with inaccuracy. The current understanding is that distinct error-prone polymerases may preferentially replicate past distinct lesions. The use of these error-prone polymerases is carefully regulated during DNA repair since they provide a balance between continuing replication with mutagenic potential (see Sale et al., 2009, for a more detailed overview of this process).

HOW THE IMMUNE RESPONSE CREATES BASE DAMAGE AND SUBVERTS BER TO CREATE VARIATION

Although spontaneous deamination of deoxycytidine to deoxyuridine is relatively infrequent, this process is enhanced in B cells during the development of the immune response by activation-induced cytidine deaminase (AID) to promote antibody gene diversification via the processes of somatic hypermutation (SHM) or CSR (Muramatsu et al., 2000; Neuberger et al., 2003; Petersen-Mahrt et al., 2002). Further, as described above, although the BER machinery can efficiently repair deoxyuridine residues with exquisitely high efficiency in somatic cells to limit mutational changes, the immune response subverts the processing of the deoxyuridine residues in B cells to enhance antibody gene diversification by exploiting the process of TLS or by promoting DSB formation. The core processes of SHM and CSR are described in Chapter 27. Here, we will specifically consider the process in the context of genomic instability and chromatin disorders. SHM involves the creation of missense mutations and, less frequently, deletions or insertions at high frequency into the V regions of the immunoglobulin genes. The uracil residues generated by deamination of cytidine either spontaneously or via AID are recognized by a specific uracil-recognizing glycosylase termed uracil-N-glycosylase 2 (UNG2) (UNG1 is an additional uracil glycosylase that is specifically expressed in the mitochondria). The current model is that AID deaminates cytosine to deoxyuridine residues on single-stranded DNA, which, following removal by UNG2 and the action of an apyridiminic endonuclease (APE1) on the abasic site, leads to the formation of an SSB. Although such SSBs can be readily repaired in somatic cells, their processing during SHM appears to involve the use of the error-prone polymerases η and ζ in a modified process of TLS (Sale et al., 2009). Interestingly, the mismatch correction complex, MSH2/MSH6, is also required for SHM. A potential role of this complex is the recruitment of exonuclease I (Exo1), to enlarge the gap and create the possibility of further mutational changes, and the error-prone polymerases (Xue et al., 2006).

CSR, the process of DNA recombination between two different switch (S) regions upstream of the immunoglobulin (Ig) constant (C) region encoding genes, generates isotypic variation from IgM. CSR involves the generation of a DSB via two AID-induced deamination events. Although the precise mechanism leading to a DSB is unclear, the generation of such an intermediate is strongly suggested by the observation that ATM-dependent damage-response signaling is activated during CSR in an AID-dependent manner, with the subsequent recruitment of the ATM-dependent signaling proteins, including the MRN complex, MDC1, and 53BP1 (Petersen et al., 2001). Moreover, γH2AX formation occurs during the process (Petersen et al., 2001). Further, mice lacking these damage-response proteins display defects in CSR (Celeste et al., 2002; Lou et al., 2006; Ward et al., 2003). Further support for the notion that CSR involves a DSB intermediate is the observation that the NHEJ enzymes are also required for the process, most likely carrying out the final rejoining step of CSR (see Durandy, 2009, for a review).

The mechanism of SHM and CSR has been further supported by the characterization of patients defective in these two processes. Patients deficient in AID have been described and will be considered in Chapter 27. As predicted, such patients show defective SHM and CSR but do not show genomic instability, since AID is specifically expressed in B cells, and additionally also likely reflects the redundancy of the glycosylases. A small number of UNG-deficient patients have also been reported who exhibit a hyper-IgM syndrome typical of impaired CSR (see Chapter 27).

In contrast, A-T and NBS patients show genomic instability in addition to defects in CSR (described in Chapters 47 and 48), consistent with the notion that the damage-signaling process is activated uniquely following DSB formation. Another disorder, (RIDDLE syndrome [radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties]) has been described with an immunodeficiency phenotype entirely consistent with impaired CSR (Stewart et al., 2007). Thegenetic defect in this condition was identified as lying in the ubiquitin ligase, RNF168, which was subsequently identified to be a critical component of the ATM-dependent damage-signaling response responsible for the recruitment of 53BP1 to the DSB (Stewart et al., 2009). Cell lines derived from this RIDDLE syndrome patient display an impaired ability to recruit 53BP1, radiosensitivity, and genomic instability. As mentioned, the precise function of the damage-response proteins in CSR remains unclear, however. Although 53BP1 and MDC1 are essential for CSR, they are only required for the repair of a subset of DSBs induced by ionizing radiation (Riballo et al., 2004).

Finally, since patients lacking the core NHEJ proteins usually present with dramatically reduced T- and B-cell numbers, an overt defect in CSR cannot be readily observed. However, one study using mice with a hypomorphic mutation in *DNA ligase IV* strongly suggested there was a further decrease in CSR (Nijnik et al., 2009).

IMPACT OF CHROMATIN STRUCTURE ON DNA DAMAGE RESPONSES AND IMMUNODEFICIENCY

There is increasing evidence to suggest that the core histones can hinder the access of critical DNA processing enzymes, including those required for repair, transcription, and replication, and that they are displaced or mobilized at the sites of DNA damage. The precise mechanisms underlying such chromatin remodeling are only just emerging, albeit with amazing alacrity (for a review see van Attikum and Gasser, 2009). To date, these studies have been best characterized in yeast, but the process is now being dissected in mammalian cells. Two critical complexes in this context are IN080 and SWRI (van Attikum and Gasser, 2009). Additionally, a subfraction of DNA is tightly compacted as heterochromatic DNA via a range of DNA and histone modifications as well as the binding of specific proteins such as heterochromatin protein 1 (HP1) and Kap1. DNA and histone methylation represent important classes of modification associated with

heterochromatinization as well as histone acetylation (for a review see Cedar and Bergman, 2009). Heterochromatin can be subdivided into constitutive and facultative heterochromatin, with the former representing regions such as pericentric DNA that is compacted in all cell types and the latter representing regions that may be compacted in a developmentally regulated manner (Trojer and Reinberg, 2007). Generally, heterochromatinized DNA is more transcriptionally inactive than euchromatic DNA sequences, although transcriptionally active or silent sequences can be located in both subtypes of DNA.

Recent studies have provided strong evidence that the higher-order DNA structure has a strong impact upon DSB repair and signaling (Goodarzi et al., 2008). As discussed above, in mammalian cells, DSBs are repaired with fast and slow kinetics, and the slow component of DSB repair has been shown to represent the repair of DSBs located within or close to heterochromatic (HC) DNA (Goodarzi et al 2008). Further, the repair of HC-DSBs appears to require ATM and ATM-dependent damage-response signaling proteins as well as the core NHEJ proteins. The available evidence suggests that the critical HC building factor, Kap1, is phosphorylated by ATM after DNA damage and that this affects its chromatin-binding capacity, potentially enhancing relaxation of the compacted HC in the vicinity of the DSB. This step appears to be a prerequisite for repair of DSBs located within HC regions. Indeed, multiple laboratories have reported that HC serves as a barrier to γH2AX signal expansion (Kim et al., 2007; Kruhlak et al., 2007). Taken collectively, the findings suggest that a critical role of ATM might be to overcome the barrier to DSB repair and signaling that is posed by higher-order chromatin structure. Moreover, ATM likely has a broader impact on chromatin architecture after DNA damage; indeed, mounting evidence suggests that it effects significant and broad changes to the chromatin in the vicinity of a DSB. It is thus highly likely that ATM has a similar role during immunodevelopment, although the precise details have not yet been elucidated (Fig. 46.3).

Although several disorders that have an impact upon higher-order chromatin structure have been described, immunodeficiency with centromere instability and facial anomalies (ICF) is of most relevance in the present context, since an impaired immune response is a prominent clinical feature of ICF syndrome and, indeed, many patients succumb to infections at an early age (see Chapter 49). ICF will be introduced here in the context of its overlap with genomic instability disorders. A diagnostic feature of ICF syndrome is hypomethylation of the classical satellite repeat sequences, which are the pericentromeric satellite regions on chromosomes 1, 9, and 16 (Probst and Almouzni, 2008). These regions are normally heavily methylated in an epigenetic context and are specifically hypomethylated in ICF syndrome (Hassan et al., 2001). Two DNA methyl transferases, MT3a and MT3b, which are encoded by *DNMT3a* and *b*, respectively, have been shown to

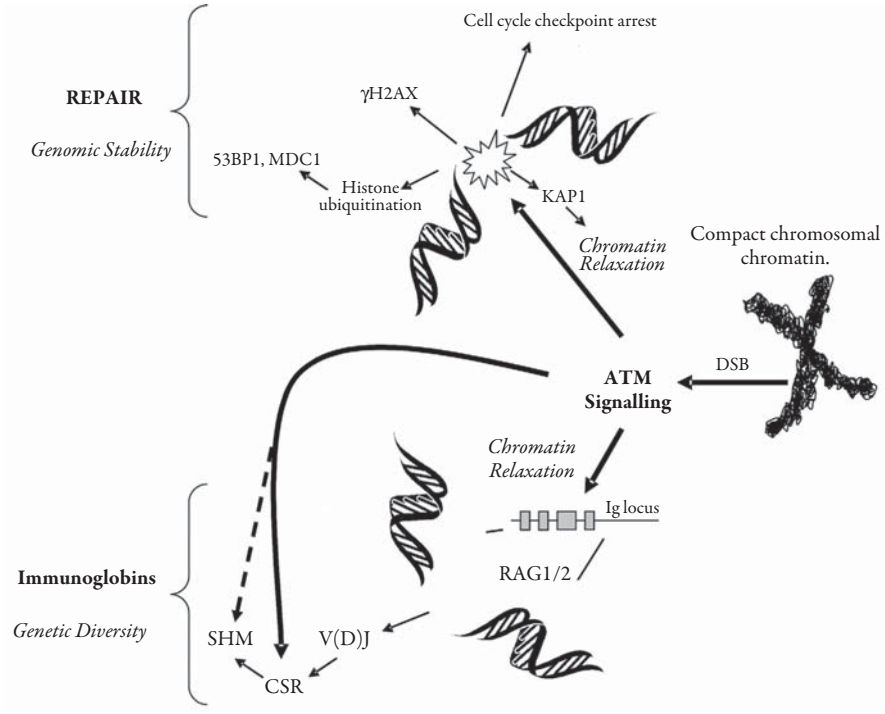


Figure 46.3 *The impact of chromatin structure and ATM damage-response signaling on the DNA damage response and immune development.* DNA is compacted by histone binding and an array of DNA and histone modifications, as well as repressor proteins. This compaction hinders the DNA damage response and developmental processes, including immune development. Increasing evidence points to ATM playing a central role in promoting chromatin changes to enhance the DNA damage response. Current studies suggest that it may play a similar role during immune development. The figure highlights potential roles of ATM signaling during these processes, although the precise contribution of ATM signaling is still emerging.

contribute specifically to these methylation events while not affecting other classes of methylation (Okano et al., 1999). It is now a decade since pathogenic mutations in *DNMT3B* have been described in some ICF patients (Hansen et al., 1999; Okano et al., 1999). Consistent with the notion that ATM is required to relieve the barrier posed by heterochromatin DNA to DSB repair and that in the ICF syndrome heterochromatin is more relaxed, ICF syndrome cells have been shown to have a less marked requirement for ATM for DSB repair (Goodarzi et al., 2008).

ICF syndrome patients have impaired humoral and cellular immunity with low levels of immunoglobulins IgG, IgA, and IgM, decreased numbers of CD3-, 4-, and 8-positive cells, and impaired lymphoproliferation following phytohemagglutinin A (PHA) or pokeweed mitogen (PWM). Exactly how these changes in methylation status of the satellite DNA sequences affect immune development is currently unclear. However, given that the ATM-dependent DNA damage-response signaling proteins serve to relax the chromatin structure to facilitate processing of DNA ends, and given the requirement of these proteins for CSR, it is possible that a relaxed or less compacted chromatin structure is required to ensure that appropriate processing can occur during CSR. There is also mounting evidence that methylation changes take place during immune development to allow the transcriptional activation of specific genes during CSR and that aberrant transcriptional regulation in ICF may affect lymphocyte development. Also, the timing of replication is regulated by heterochromatic status, and alterations in the regulation of replication may contribute to the features of this disorder (Ehrlich et al., 2008; Jin et al., 2008).

IMPACT OF THE DNA DAMAGE RESPONSE AND HIGHER-ORDER CHROMATIN STRUCTURE ON OTHER DEVELOPMENTAL PROCESSES

Both ICF and the genomic instability disorders display other characteristic features in addition to immunodeficiency, which result from a broader role of the defective pathway or gene during development. A brief overview of these roles will be considered here since they represent important factors in the differential diagnosis of many of these conditions but also need to be carefully considered in optimizing patient care.

CANCER PREDISPOSITION

As mentioned above, DNA is constantly subjected to damage, and a critical role of the proteins that function in DDR is the maintenance of genomic stability. Cells with unrepaired DSBs show increased lethality. Thus, cell loss can represent an important contribution to the clinical manifestation of DDR defects. However, in most cases the defect in the genomic instability disorders decreases rather than completely blocks DSB repair. DSBs that are not repaired in a timely fashion frequently undergo misrepair, allowing the proliferation of cells with genetic changes. Additionally, loss of the ATM signaling pathway impairs cell-cycle checkpoint arrest and apoptosis, processes that serve to prevent the proliferation of damaged cells (Lavin, 2008). Thus, tumor predisposition is an important phenotype observed in some of the DDR defective syndromes.

Artemis and the core NHEJ proteins are dispensable for both cell-cycle checkpoint arrest and apoptosis, in marked distinction to ATM. Indeed, the most consistent consequence of loss of NHEJ proteins is elevated cell death after agents that induce DSBs, demonstrating the significance of an unrejoined DSB as a lethal lesion. Since DNA ligase IV is essential, the mutations causative of LIG4 syndrome are hypomorphic and residual, but slow DSB rejoining remains (O'Driscoll et al., 2001). XLF/Cernunnos patients also show substantial residual DSB rejoining activity (Riballo et al., 2009). Most LIG4 syndrome and XLF/Cernunnos-deficiency patients display CID rather than SCID, providing further support for the notion that residual V(D)J recombination occurs. Strikingly, leukemia or lymphoma is observed in approximately 25 percent of LIG4 syndrome patients and, perhaps not surprisingly, is more marked in patients with less severe immunodeficiency, most likely due to the higher number of target T and B cells present (O'Driscoll and Jeggo, 2006). One possible explanation is that aberrant V(D)J recombination promotes oncogene expression and enhances the onset of lymphoid tumors. An alternative explanation is that the impaired immune response attenuates immune surveillance, allowing Epstein-Barr virus (EBV)–type infections and hence the onset of EBV-induced tumorigenesis. Consistent with this possibility, most, although crucially not all, tumors are of B-cell origin. Artemis, which, in contrast to DNA ligase IV, is nonessential since it is required for only a subset of DSB repair, is essential for V(D)J recombination due to its requirement in hairpin cleavage (Ma et al., 2002). Because Artemis patients harboring null mutations are T^- and B^- SCID, they do not have target cells for the induction of lymphoma or leukemia onset. Consequently, predisposition to lymphoid tumors is not common in radiation-sensitive SCID patients due to Artemis mutations. Patients with hypomorphic Artemis mutations have been described, however, and a predisposition to leukemia and lymphoma has been reported among these patients (Ege et al., 2005; Evans et al., 2006; Moshous et al., 2003). Such tumors are frequently of EBV origin (Moshous et al., 2003). ATM and NBS patients have a very high predisposition to tumors of lymphoid origin, which may be a consequence of an impaired fidelity of V(D)J recombination or CSR and/or the inability to remove damaged cells. ICF has not been reported as a cancer predisposition syndrome to date, although currently the number of patients and their early death from infections precludes a realistic evaluation of cancer predisposition. It will be interesting to evaluate this as patient care improves and increases the life expectancy of affected individuals.

NEURONAL DEVELOPMENT

A further marked characteristic of damage-response disorders is impaired neuronal development and/or enhanced neurodegeneration (O'Driscoll and Jeggo, 2008b). In most instances,

including LIG4 syndrome, XLF/Cernunnos deficiency, and NBS, this is manifest as the occurrence of microcephaly at birth. The microcephaly is rarely progressive postnatally. This feature is also frequently observed in other DDR disorders that are not associated with immunodeficiency, most strikingly in ATR-Seckel syndrome and Blooms syndrome (O'Driscoll et al., 2003). In marked contrast, patients with A-T and A-T–like disorders (due to mutations in the *MRE11* encoding component of the MRN complex) display progressive ataxia, which is normally manifest in the early years of childhood but not immediately evident at birth. Loss of Artemis, in contrast, does not confer any overt neuronal development (Moshous et al., 2001). The most likely explanation for these differences is that NHEJ represents a critical process required for the propagation of neuronal stem cells or their early progenitors. The embryonic neuronal stem cells, the ventricular-zone cells, undergo a high rate of cell division during embryogenesis, and since these stem cells disappear shortly after birth, loss of early progenitor cells cannot be readily regained. It is possible that the rapid cell division of the ventricular-zone cells during embryogenesis results in high oxidative damage or that DSBs arise during this rapid phase of replication. Thus, DSBs generated either via oxidative damage or replication stress may have a critical requirement for NHEJ for their repair. Interestingly, the embryonic brain is also highly sensitive to radiation, consistent with the possibility that unrepaired DSBs readily cause apoptosis or cell loss in this compartment. Since loss of Artemis affects only the repair of a subset of DSBs, Artemis may be less critical for this aspect of development. Indeed, Artemis-SCID patients do not exhibit microcephaly.

The basis underlying the striking difference between A-T and NBS patients in their neuronal phenotype is currently unclear. A recent study, however, raised the possibility that the mutation in NBS patients does not have an impact upon apoptosis (Shull et al., 2009). Thus, cells with unrepaired DSBs may be lost in NBS patients but not in A-T patients, where DSB-induced apoptosis does not occur. Further work, however, is required to substantiate such a model.

CONCLUDING REMARKS

Immune development and the DDR represent two processes that superficially appear to have opposite goals. Immune system development is underscored by the need to generate diversity, while the DDR serves to preserve genomic stability. Fascinatingly, the immune system co-opts elements of the DDR machinery in processes such as V(D)J recombination, CSR, and SHM as a fundamental tool to cut, splice, and alter the coding properties of DNA in a highly complex and ordered fashion to generate its requisite diversity. Consequently, human disorders, including those discussed in the subsequent chapters, caused by defective DDR frequently manifest with some form of immune dysfunction. Furthermore, disorders such as ICF syndrome, where the primary defect is in chromatin structure, provide an additional element of overlap between DNA processing and immune development. Changes to chromatin structure are essential during the response to DNA damage, and a critical role of ATM signaling appears to be to implement such changes. The role of ATM and its signaling proteins during immune development is very likely to involve chromatin alterations. Thus, the ability to modify chromatin architecture appropriately is likely to be a critical, and still understudied, step during immune development. The basis underlying the immunodeficiency observed in ICF could play a useful role in understanding the intricate balance between chromatin, DNA processing, and immune development.

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47.

ATAXIA-TELANGIECTASIA

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Ataxia-telangiectasia (A-T) (OMIM 208900) is a complex multisystem disorder characterized by progressive neurological impairment, oculocutaneous telangiectasia, variable immunodeficiency with recurrent sinopulmonary infections, cancer susceptibility, and sensitivity to ionizing radiation. First described in 1926 (Syllaba and Henner, 1926) and established as a disease entity in 1957 by Boder and Sedgwick, A-T has evolved from a rare disorder to a paradigm for cell signaling and cancer (Chen et al., 1978; Gatti et al., 1988; Gotoff et al., 1967; Lavin, 2008; Morgan et al., 1968; Peterson et al., 1963; Savitsky et al., 1995a; Taylor et al., 1975). Localization of the responsible gene *ATM* (Ataxia-Telangiectasia Mutated) to chromosome 1 1q22-23 by Gatti et al. (1988), and then identification of *ATM* by Savitsky et al. (1995a) as a member of the phosphatidylinositode 3-kinase-related protein kinase (PIKK) family genes, which are involved in cellular responses to DNA damage, cell-cycle control, and intracellular protein transport, provided a basis for understanding of the pleiotropic nature of A-T.

The disease A-T is inherited as an autosomal recessive trait with full penetrance (Ferak et al., 1968; McKusick and Cross, 1966; Tadjoedin and Fraser, 1965). A-T, represented equally in males and females, has been reported in all ethnic groups throughout the world, with a disease prevalence from 1 in 40,000 (Boder and Sedgwick, 1970) to 1 in 88,000 live births (Swift et al., 1986; Woods et al., 1990).

In this chapter, we will provide insight into the clinical, cellular, and molecular abnormalities in A-T and an overview of the *ATM* gene and the ATM protein with our current understanding of their functions and the pathways from the defective gene to the clinical manifestations.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

In the initial report of A-T, Syllaba and Henner (1926) observed progressive choreoathetosis and ocular telangiectasia in three members of a single family. In 1941, Louis-Bar described progressive cerebellar ataxia and cutaneous telangiectasia in a Belgian child, hence the name "Louis-Bar syndrome." Boder and Sedgwick (1957) and Biemond (1957), with the aid of autopsies, reported organ developmental abnormalities, neurological manifestations, and recurrent sinopulmonary infections. Later on, several additional reports confirmed the major manifestations of this syndrome (Boder and Sedgwick, 1958; Centerwall and Miller, 1958; Sedgwick and Boder, 1960; Wells and Sly, 1957). The main clinical features of the disease are outlined in Table 47.1 (Boder, 1985; Boder and Sedgwick, 1963).

CEREBELLAR ATAXIA

In A-T, it is evident from the earliest reports that neurological features are prominent (Boder, 1985). Ataxia, generally the presenting symptom in this syndrome, becomes apparent when a child begins to walk at the end of the first year of life, by ataxic gait and truncal movements. Similar to other major characteristics of A-T, ataxia is progressive, affecting the extremities and then the speech. Eventually, involuntary movements dominate, and the child may require a wheelchair by the end of the first decade of life. However, there are some recent reports of rare patients who had slow progression of ataxia (Dörk et al., 2004) or manifest neurological findings only in adulthood (Saviozzi et al., 2002; Sutton et al., 2004).

Table 47.1 CLINICAL FEATURES IN ATAXIA-TELIANGIECTASIA (A-T)

CLINICAL FEATURE	CASES WITH FEATURE (NO.)	CASES WITH DATA AVAILABLE (NO.)
I. Neurological abnormalities		
Cerebellar ataxia, infantile or childhood onset	101	101
Diminished or absent deep reflexes	54	61
Flexor or equivocal plantar response	60	61
Negative Romberg sign	28	36
Intact deep and superficial sensation	51	52
Choreoathetosis	61	67
Oculomotor signs		
Apraxia of eye movements	47	56
Fixation of gaze nystagmus	48	58
Strabismus	7	15
Dysarthric speech	70	70
Drooling	43	49
Characteristic facies and postural attitudes	60	61
II. Telangiectasia, oculocutaneous	101	101
III. Frequent sinopulmonary infection	60	72
IV. Familial occurrence	43	96
V. Reported mental deficiency	22	66
VI. Equable disposition	34	34
VII. Retardation of somatic growth	42	58
VIII. Progeric changes of hair and skin	46	52

SOURCE: From Boder (1985).

The underlying pathology appears to be primarily progressive cerebellar cortical degeneration. The neuropathological features of A-T are comprehensively detailed in two reviews by Boder (1985) and Sedgwick and Boder (1991). Cortical cerebellar degeneration involves primarily Purkinje and granular cells, but basket cells are also affected. While degenerative changes in the brain are seen predominantly in the cerebellum, it is clear from an increasing number of autopsies that changes to the central nervous system (CNS) in A-T are more widespread. Degenerative changes in the dentate and olivary nuclei were noted in early studies, and these observations were extended to include changes in the spinal cord and spinal ganglia, the cerebrum, the basal ganglia, and the brain stem, especially in older patients. Amromin et al. (1979) have described distinctive gliovascular abnormalities in the cerebral white matter and also in the brain stem and spinal cord. These consist of dilated capillary loops, many with fibrin thrombi, with perivascular hemorrhages and hemosiderosis, surrounded by demyelinated white matter, reactive gliosis, and numerous atypical astrocytes (Boder, 1985). These vascular changes are seldom seen in the basal ganglia or cerebral cortex and have not been reported in the cerebellum. Since they are seen only in older patients, it is unlikely that they are a primary cause of cerebellar or spinal cord degeneration.

TELANGIECTASIA

The second major clinical feature of the disease is telangiectasia. In general, it has a later onset than ataxia, between 2 and 8 years of age (Boder, 1985; McFarlin et al., 1972). However, telangiectasia can be detected much earlier, particularly with a high index of suspicion in children with a family history of A-T. Telangiectasia represents a dilation of blood vessels, primarily in the ocular sclerae, and often gives the impression of bloodshot eyes (Color Plate 47.I). The telangiectasias may also appear in the butterfly area of the face and as hairline telangiectasias on the ears. Patches of telangiectasia elsewhere in the skin are less common. Ocular telangiectasias may be mistaken for conjunctivitis but can be readily distinguished because they are characterized by dilated vessels against a white background, whereas in conjunctivitis the background is pink. It is not clear how the defective ATM protein would give rise to telangiectasia. It has been suggested that the blood-vessel abnormality represents a progeric change, as it mimics telangiectasias in normal aged individuals (Boder, 1985). The occurrence of telangiectasias in tissue of normal individuals undergoing radiotherapy may also be related to the telangiectasia in A-T. Since hypersensitivity to radiation and to compounds capable of generating active oxygen radicals is a characteristic of this disease, a decreased ability to cope with endogenously generated damage from oxygen radicals may be a factor in the development of telangiectasias in A-T. Indeed, there is now good evidence that A-T cells are in a state of oxidative stress (Barlow et al., 1999; Cosentino et al., 2011; Gatei et al., 2001a; Guo et al., 2010; Kamsler et al., 2001; Patel et al., 2011; Watters et al., 1999). However, light or sun damage may also be contributing to telangiectasias in the eyes and on the skin.

PREDISPOSITION TO SINOPULMONARY INFECTION

The third major feature of A-T is susceptibility to infections. Recurrent infections are reported in up to 80 percent of patients in some studies (Waldmann, 1982). Infections become apparent by the age of 3 (Andrews et al., 1960; Centerwall and Miller, 1958; McFarlin et al., 1972). Major sites of infection are the upper and lower respiratory system. The infections include otitis and sinusitis as well as recurrent pneumonia, which may progress into bronchiectasis and pulmonary fibrosis severe enough to cause clubbing of fingers and toes, and, eventually, respiratory insufficiency and death (Sedgwick and Boder, 1991). Boder and Sedgwick (1963) have reported frequent respiratory infections in 83 percent of cases, and chronic bronchitis with or without bronchiectasis

in 52 percent of cases. Patients with A-T are particularly susceptible to common bacterial pathogens and viruses but do not appear to be subject to generalized or persistent fungal or protozoan infections (McFarlin et al., 1972; Nowak-Wegrzyn et al., 2004; Peterson and Good, 1968).

McFarlin et al. (1972) demonstrated a correlation between the severity of the respiratory infections and reduced immune responses. The observation of Boder and Sedgwick (1957) that the thymus is absent or poorly developed in A-T, together with reports of hypogammaglobulinemia (Gutmann and Lemli, 1963; Williams et al., 1960), suggested a basis for the infection susceptibility. It is now known that there is a more generalized defect in multiple aspects of the immune response in A-T patients (Exley et al., 2011; Waldmann, 1982).

IMMUNODEFICIENCY

Immunodeficiency in A-T is highly variable, involving both antibody production and cellular immunity (McFarlin et al., 1972; Peterson et al., 1963). Some patients have a history of chronic sinopulmonary infections, others have recurrent infections, and yet another group may show no higher incidence of infections than their unaffected siblings (McFarlin et al., 1972; Nowak-Wegrzyn et al., 2004).

ANTIBODY-MEDIATED IMMUNITY

The presence of hypogammaglobulinemia has been equated with the high frequency of infections in A-T (Andrews et al., 1960; Centerwall and Miller, 1958; Gutmann and Lemli, 1963; Williams et al., 1960). The first insight into the nature of the immune defect was provided by Thieffry et al. (1961), who showed that serum IgA was absent in a number of patients with A-T. It was subsequently found that up to 80 percent of patients had low or undetectable serum IgA levels (Boder, 1975; Epstein et al., 1966; Ersoy et al., 1991; McFarlin et al., 1972). IgE deficiency has been reported with almost the same frequency (Ammann et al., 1969; McFarlin et al., 1972). In a smaller percentage of patients, IgG, especially IgG2, is reduced (Oxelius et al., 1982). The presence of low-molecular-weight IgM has also been reported in up to 80 percent of patients (McFarlin et al., 1972; Stobo and Tomasi, 1967). Serum IgD concentrations in A-T were found to be significantly elevated compared to the control group in one study (Sanal et al., 1998). The abnormalities in immunoglobulin levels in A-T are not due to reduced B-cell numbers but appear to result from a defect in B-cell differentiation (Gail-Peczaska et al., 1973; Lawton et al., 1972). A spectrum of responses to bacterial antigens, from normal to severely reduced, is observed in A-T patients (McFarlin et al., 1972; Sanal et al., 1999, 2004; Sedgwick and Boder, 1972). On the other hand, natural antibody and antibody responses to challenges with viral antigens are dramatically decreased compared with those of controls (Waldmann, 1982). Circulating autoimmune antibodies against immunoglobulin, muscle, and mitochondria may also be found in A-T patients (Ammann and Hong, 1971). Although the deficiency in IgA and IgE is primarily due to a reduced rate of synthesis, autoimmune antibodies against these molecules may also cause an increase in their destruction rates (Strober et al., 1968; Waldmann, 1982).

An intrinsic defect in the maturation of B cells and/or reduced T-cell helper activity was suspected in A-T. Since two of the chromosomes that contain the immunoglobulin and T-cell–receptor genes, chromosomes 14 and 7, respectively, show frequent breaks, rearrangements, and translocations in lymphocytes from A-T patients, a defect in gene regulation or in the recombination mechanisms required to generate mature B- and T-cell–receptor genes was suspected earlier (Cohen et al., 1975; Hecht et al., 1973; Taylor et al., 1976. Now, it is known that, in A-T, there is a defect in class-switch recombination (CSR) (Bredemeyer et al., 2008; Kracker et al., 2010; Kracker and Durandy, 2011; Pan-Hammarström et al., 2003, 2006; Perkins et al., 2002; Reina-San-Martin et al., 2004; Zha et al., 2011). Being a DNA damage sensor, ATM, along with NBS1, is activated by the DNA breaks in the switch (S) regions during CSR. ATM phosphorylates a large number of substrates that activate the DNA damage response, including histone H2AX. Phosphorylated H2AX, called γH2AX, leads to recruitment of other DNA damage-response factors, which eventually results in effective CSR. Therefore, in the setting of ATM deficiency, chromosomal VDJ recombination is disturbed and the process of immunoglobulin class switch is impaired. In fact, patients with A-T commonly have normal or elevated serum levels of IgM (Ersoy et al., 1991; Exley et al., 2011). There are even reports of patients with A-T initially diagnosed with hyper-IgM syndrome, in whom the diagnosis of A-T was established later by identification of missense mutations in the *ATM* gene (Aghamohammadi et al., 2010; Pietrucha et al., 2010).

CELL-MEDIATED IMMUNITY

Faulty development of the thymus is very characteristic of A-T (Fireman et al., 1964; McFarlin et al., 1972; Peterson et al., 1964b). The thymus often cannot be identified grossly at autopsy, but is only recognized microscopically as a scattered collection of thymic reticular elements with a marked paucity of thymocytes and absence of Hassell's corpuscles and corticomedullary demarcations. These abnormalities reflect a developmental defect rather than atrophy of the thymus (Peterson et al., 1964b). Approximately one third of patients with A-T have unequivocal lymphocytopenia, which is usually mild in degree (Roifman and Gelfand, 1985). The proportion of lymphocytes bearing T-cell markers is reduced, and cells with receptors for IgM may also be diminished in number (Waldmann, 1982). In vivo and in vitro functional activity of T cells is variable; however, when considered as a group, A-T patients are deficient in their responses (Epstein et al., 1966; Ersoy et al., 1991; Fireman et al., 1964; McFarlin et al., 1972). Variability is evident in delayed-type hypersensitivity skin test response to phytohemagglutinin (PHA) and to antigens such as mumps, tuberculin purified protein derivative (PPD), and dinitrochlorobenzene as well as in in vitro lymphocyte proliferation to PHA and pokeweed mitogen (PWM). Delayed rejection of a skin graft from a human leukocyte antigen (HLA)-incompatible donor has been described in up to

80 percent of A-T patients (Waldmann, 1982). O'Connor and Scott-Linthicum (1980), who demonstrated that bound PHA could be efficiently internalized by A-T lymphocytes, proposed that a defect existed at the intracellular signaling level.

Disruption of the *Atm* gene in mice gives rise to a phenotype that largely reflects that seen in the human disease (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). Immune defects observed in $Atm^{-/-}$ mice include a smaller size of lymphoid tissues, a reduction of CD4 and CD8 single-positive T lymphocytes, an increase in premature double-positive mature T lymphocytes, greatly reduced peripheral T cells, and defective T-cell responses. Productively rearranged TCRαβ gene is required for thymocyte proliferation and rearrangement for the transition of double-positive to single-positive T cells (Shinkai et al., 1993; von Boehmer, 1994). Since $Atm^{-/-}$ mice were defective in both processes, Chao et al. (2000) introduced a functional TCRαβ transgene into these mutant mice. This approach led to the rescue of defective T-cell differentiation and partial rescue of thymus hypoplasia, indicating that positive selection of thymocytes is normal in $Atm^{-/-}$ mice. It is also of interest that when quiescent mature T cells are exposed to DNA intercalating agents, they readily undergo apoptosis in an Atm-dependent manner (Bhandoola et al., 2000). Immature thymocytes, by contrast, are resistant to apoptosis induced by this DNA damage-signaling pathway, even though double-strand breaks (DSBs) are present in DNA. This resistance may be due to downregulation of this pathway in immature cells, consistent with the observation that freshly prepared peripheral blood mononuclear cells from humans have low levels of ATM protein and a low basal level of ATM kinase (Fukao et al., 1999).

The CD4/CD8 ratio in A-T patients is reversed compared with controls because of a decrease in the total number of CD4 T cells (Fiorilli et al., 1983). Furthermore, A-T patients have a relative increase in T cells bearing γ/δ antigen receptors compared with those with α/β receptors, unlike the T-cell populations in most other immunodeficiency syndromes. Carbonari et al. (1990) suggested that a defect in recombination might explain the defects in both T-cell and B-cell differentiation more than 20 years ago. Such a defect could also account for the high incidence of chromosomal rearrangements involving primarily chromosomes 7 and 14 (Aurias et al., 1980). Four common sites of chromosome breakage have been described in A-T patients: 7p14, 7q35, 14q11.2, and 14q32. Since the T-cell–receptor genes and the Ig heavy-chain genes map to these sites (Hedrick et al., 1984; Sim et al., 1984), a defect in DNA recombination could explain the immunodeficiency, the hypersensitivity to radiation, and the lymphoid malignancies seen in A-T. In support of this concept, Meyn et al. (1993) demonstrated spontaneous intrachromosomal recombination rates 30 to 200 times higher in A-T fibroblasts than in fibroblasts from controls. This observation was not confirmed using extrachromosomal test DNA substrates (Hsieh et al., 1993). Taken together, these studies suggested that a defect in intrachromosomal recombination could contribute to genetic instability and predisposition to cancer as well as cellular immunodeficiency in A-T. Now, it is apparent that ATM plays a major role in maintaining TCR-α expression during the recombination process (Matei et al., 2007), and its deficiency impairs thymocyte maturation because of defective resolution of TCR-α locus coding end breaks (Vacchio et al., 2007). Finally, it has been discovered that ATM is instrumental in the repair of physiological DSBs in the processes of Ig CSR and B- or T-cell–receptor V(D)J recombination in lymphoid cells (Kracker and Durandy, 2011), providing a better explanation into the characteristic features of A-T.

Immunodeficiency in A-T has been proposed to resemble premature aging of the immune system by multiple cellular and molecular immune parameters (Exley et al., 2011). In A-T, a relative deficiency of naïve $CD19^+IgD^+$ B cells, and classical naïve $CD4^+$ $CD45RA^+$, thymic naïve $CD4^+CD31/45RA^+$, and naïve $CD8^+CD27/28/45RA^+$ T cells has been observed. No deficit has been seen in $CD56^+$ NK cells. Thus, immunodeficiency in A-T particularly affects naïve B and T cells, which is a typical feature in physiological aging.

CANCER PREDISPOSITION

One of the major hallmarks of A-T is the predisposition to develop malignancies, most lymphoid in origin (Boder and Sedgwick, 1963). The association between a defective thymus, immunodeficiency, and the high frequency of lymphoid malignancy became evident early (Leveque et al., 1966; Miller, 1967; Peterson et al., 1964a). Chromosome instability as an explanation for the increased incidence of malignancy was proposed with the observation that leukemic cells from an A-T patient had a translocation involving chromosomes 12 and 14 (Hecht et al., 1966). Regular monitoring of this patient revealed a progressive increase in translocation-bearing, abnormal lymphocytes to 78 percent of the total lymphocytes before the patient succumbed to infection (Hecht et al., 1973). However, while translocations involving chromosome 14 have been associated with malignancy in Burkitt's lymphoma (Klein, 1981), patients with A-T without signs of leukemia or lymphoma may have clonal expansion of cells with chromosome 14/14 translocations to as much as 80 percent of the total lymphocytes (Al Saadi et al., 1980; Beatty et al., 1986).

Lymphoid malignancies in A-T are of both B-cell and T-cell origin and include non-Hodgkin's lymphoma, Hodgkin's lymphoma, and several forms of leukemia (Hecht and Hecht, 1990; Spector et al., 1982). At autopsy, non-Hodgkin's lymphoma accounted for approximately 40 percent of neoplasms detected, leukemias about 20 percent, and Hodgkin's lymphomas 10 percent. The increased frequency of lymphoid tumors in A-T could be accounted for by a defect in immune surveillance as part of the underlying immunodeficiency; however, the picture is more complex, as malignancies are not confined to the lymphoid system. In an analysis of 108 A-T patients with 119 neoplasms, Hecht and Hecht (1990) reported that 31 of these (26 percent) were solid tumors varying in type and location. Determination of subsequent risk in A-T

patients diagnosed with one type of neoplasm revealed that approximately 25 percent of patients with solid tumors subsequently developed non-Hodgkin's lymphoma or leukemia. A very low risk of subsequent neoplasms existed when the first tumor was lymphoid in origin. In a retrospective study in the United States, mortality from all causes in A-T was 50-fold and 147-fold higher for white and black A-T patients, respectively, than expected, based on overall U.S. mortality rates (Morrell et al., 1986). Among 263 A-T patients, there were 52 primary cancers—a 61-fold cancer excess for white probands and a 184-fold excess for black probands. Morrell et al. (1986) also found that the cancer excess was most pronounced for lymphoma, with 252-fold and 750-fold excesses observed for whites and blacks, respectively. As a cause of death in A-T, neoplasia is the second most frequent after pulmonary disease. Of 62 complete autopsy reports (Sedgwick and Boder, 1991), 29 deaths (47 percent) were caused by pulmonary complications, 14 (22 percent) by malignancy, and 16 (26 percent) by a combination of both. The lifetime cancer risk among A-T patients has been estimated to be between 10 and 38 percent (Morrell et al., 1990; Spector et al., 1982).

Recently, in patients with oral cancer, one of the polymorphisms in ATM, rs189037, has been found to be more prevalent compared to controls (Bau et al., 2010). A study by Lee et al. (2011) has shown that reduced *ATM* mRNA expression in laryngopharyngeal tumors is correlated with poor outcome, implying a role for ATM in solid tumor development and host response.

PHENOTYPIC HETEROGENEITY

Phenotypic heterogeneity has long been recognized in both the clinical and laboratory features of A-T. Susceptibility to pulmonary infections, presence and degree of mental retardation, and predisposition to leukemia were used as a tentative classification of subgroups (Hecht and Kaiser-McCaw, 1982). Initially, genetic heterogeneity underlying this phenotypic variability was proposed with the description of four cellular complementation groups (Chen et al., 1984; Jaspers and Bootsma, 1982; Murnane and Painter, 1982). However, identification of *ATM* (Savitsky et al., 1995a) made it clear that all complementation groups with a classical A-T phenotype represented mutations in the same gene.

In addition, there are separate genetic disorders resulting from defects in a DNA damage-response pathway that is functionally linked to ATM, which share phenotypic features with classical A-T (Nahas and Gatti, 2007) The first, Nijmegen breakage syndrome (NBS), shares the radiosensitivity, immunodeficiency, and cancer predisposition of A-T; unlike A-T, NBS involves microcephaly with various degrees of mental deficiency, and patients do not have ataxia or telangiectasias (Curry et al., 1989; Digweed et al., 1999; Digweed and Sperling, 2004; Jaspers et al., 1988a, 1988b). The link between A-T and NBS became more clear with the identification of NBS1 (nibrin), the product of the gene defective in NBS that is functionally linked to ATM (Carney et al., 1998; Matsuura et al., 1998; Varon et al., 1998) (Table 47.2).

The second disease, A-T–like disorder (ATLD), was first described by Taylor et al. (1993) in a family in which two first cousins presented with some A-T features. The clinical course of the disease was mild. Unlike classical A-T patients, these cousins were still ambulatory in their third decade of life. Both had progressive unsteadiness in walking, dysarthria, drooling, vertical nystagmus, and intention tremors, all characteristic of A-T, but there was no evidence of telangiectasia, and immunoglobulin and α-fetoprotein (AFP) levels were normal. Stewart et al. (1999) identified the gene responsible

Table 47.2 **COMPARISON OF ATAXIA-TELANGIECTASIA (A-T), ATAXIA-TELANGIECTASIA-LIKE DISORDER (ATLD), AND NIJMEGAN BREAKAGE SYNDROME (NBS)**

A-T	ATLD	NBS
ATM-null mutations	*MREII* hypomorphic mutations	*NBS1* hypomorphic mutations
Immunodeficiency	Immunodeficiency	Immunodeficiency
Neurodegeneration	Neurodegeneration	Neurodegeneration
Telangiectasia	—	Telangiectasia
—	—	Microcephaly
—	No evidence	Mental retardation
Radiosensitivity	Radiosensitivity	Radiosensitivity
Chromosomal translocations	Chromosomal translocations (+/–)	Chromosomal translocations
Radiosensitivity	Radiosensitivity (intermediate)	Radiosensitivity
Defective cell cycle checkpoints	Intermediate defect in cell-cycle checkpoints	Defective cell-cycle checkpoints
Defective DSB repair	?	Defective DSB repair
No ATM activity	Normal ATM basal activity, defective ATM activation	Normal basal ATM activity, defective ATM activation?
Genomic instability	Genomic instability	Genomic instability
Cancer predisposition	?	Cancer predisposition

for ATLD as *MRE11* that encodes the MRE11 protein, which is part of the hMrell/Rad50/Nbsl (MRN) complex that senses DSBs in DNA (Nelms et al., 1998; Stracker et al., 2004). The *MRE11* gene locus is close to the *ATM* locus on human chromosome 11q21, and only detailed linkage analysis allows separation of these loci, necessitating analysis of the *ATM* and *MRE11* genes to distinguish between A-T and ATLD. Mutations in the *MRE11* gene lead to many of the characteristics observed in both A-T and NBS. These results provide evidence for a common involvement of both ATM and members of the MRN complex in the cellular response to DSBs in DNA.

Ataxia without telangiectasia has also been reported by Aicardi et al. (1988), so-called "Aicardi syndrome," by Maserati et al. (1988), and by Chessa et al. (1992). According to current classification, there are two types of autosomal recessive ataxias with oculomotor apraxia (AOA). In type 1 (AOA1; i.e., Aicardi syndrome), aprataxin encoded by *APTX* gene is defective (Date et al., 2001; Moreira et al., 2001). In type 2 (AOA2), there is a defect in senataxin encoded by *SETX* (Moreira et al., 2004). More recently, other DNA repair defects have been discovered in humans: a female patient with RAD50 deficiency, who phenotypically resembled NBS patients (Waltes et al., 2009), and a male patient with ubiquitin-ligase ring-finger protein (RNF168) deficiency, with A-T–like features (Devgan et al., 2011).

Even in classical A-T with ataxia and telangiectasia, the onset of clinical symptoms and the rate of progression are variable. Several reports describe differences in the age of presentation and rates of progression (Dörk et al., 2004; Hernandez et al., 1993; Sedgwick and Boder, 1991; Sutton et al., 2004). In a study of 70 A-T patients, ataxia started prior to 1 year of age in 20 percent, before 2 years in 65 percent, and by 4 years of age in 85 percent of patients (Taylor et al., 1993; Woods and Taylor, 1992). Variability in the appearance of telangiectasias, even between affected siblings, has also been reported (Sedgwick and Boder, 1991). Sanal et al. (1993) identified 30 A-T patients with variant characteristics, including severity of the ataxia, presence of telangiectasia, degree of growth retardation, mental ability, facial appearance, and immunological abnormalities.

The types and frequencies of spontaneous chromosome aberrations including translocations and inversions, largely involving chromosomes 7 and 14, are remarkably consistent in A-T patients (Taylor and Edwards, 1982). These translocations are also observed in lymphocytes from normal individuals, but at a much lower frequency. Exposure of A-T cells to ionizing radiation gives rise to increased levels of chromatid damage compared to controls (Kidson et al., 1982; Taylor et al., 1976). Comparison of lymphocytes from different A-T patients indicates that heterogeneity exists in the extent of induced damage. This is also observed when cell survival is the endpoint (Cox et al., 1978; Fiorilli et al., 1985; Jaspers et al., 1988b; Ziv et al., 1989). Chessa et al. (1992) designated 14 A-T patients from several studies as being intermediate in their response to ionizing radiation. In 13 of the 14 cases, there was a correlation between radiosensitivity as determined by chromosomal breakage and cell survival. On the other hand, radioresistant DNA synthesis ranged from normal to equally defective as in classical A-T, indicating that this parameter is less significant in causing the radiosensitivity in A-T. Twelve of the 14 patients in this study had definite ataxia and either frank telangiectasia or an increase in conjunctival vascularity.

The considerable phenotypic heterogeneity in A-T is not clearly understood even in light of the identified *ATM* gene. Several studies on *ATM* mutations predict that 70 to 80 percent give rise to truncated proteins, while the remainder represent small to large in-frame deletions and missense mutations (Byrd et al., 1996; Chun and Gatti, 2004; Gilad et al., 1996a, 1996b; Lavin et al., 2004; Savitsky et al., 1995a; Telatar et al., 1996; Wright et al., 1996). Most patients are compound heterozygotes with different mutations in the two *ATM* alleles. This information has been useful in accounting for milder forms of the disease in some A-T patients. A missense mutation in *ATM* that activates a cryptic splice/donor acceptor site resulting in the insertion of 137 nucleotides of intronic sequence (5762ins137) was present in the heterozygous state in 15 percent of A-T patients in the United Kingdom with a milder phenotype (McConville et al., 1996). A second missense mutation 7271T-G is also associated with a mild-variant A-T phenotype (Stankovic et al., 1998). Thus, allelic diversity might explain much of the heterogeneity in clinical severity in A-T. However, there are some interesting cases—for instance, two siblings with mild A-T who were found to have a truncating *ATM* mutation and severe cellular phenotype (Alterman et al., 2007). Therefore, influences other than *ATM* mutations seem to play a role in heterogeneity.

OTHER CLINICAL CHARACTERISTICS

In addition to the major clinical hallmarks, a variety of other features characterize A-T (Table 47.1). Retardation of somatic growth has been reported for about 70 percent, particularly in adolescents and older patients, whose heights are often below the third percentile. Stunting of growth may be due to the pulmonary complications, hypogonadism, and/or thymic dysplasia (Boder, 1985; Good et al., 1964). Female hypogonadism, as absence or hypoplasia of the ovaries, is quite common in A-T. This is usually associated with infantile uterus and fallopian tubes (Boder and Sedgwick, 1957; Bowden et al., 1963). Its appearance in A-T may be due to an underlying mesenchymal abnormality (Dunn et al., 1964; Miller and Chatten, 1967). Hypogonadism is also observed in male A-T patients, but to a lesser extent than in females (Boder and Sedgwick, 1958). Puberty is delayed, there is abnormal testicular histology, and spermatogenesis is impaired (Aguilar et al., 1968; Strich, 1966).

An unusual type of diabetes mellitus has been described in patients with A-T, with marked hyperinsulinism, hyperglycemia without glycosuria or ketosis, and peripheral resistance to insulin (Barlow et al., 1965; Schlach et al., 1970). Insulin resistance can partly be explained by abnormalities in the number

and affinity of insulin receptors in A-T (Bar et al., 1978). More importantly, ATM protein, being related to phosphatidylinositol 3-kinase (Jackson and Jeggo, 1995; Lavin et al., 1995; Savitsky et al., 1995a; Zakian, 1995), is involved in signal transduction and glucose transport. The kinase activity of ATM is activated by insulin through a non-DNA-damage signaling pathway to phosphorylate 4E-BP1 (PHAS-1), a regulator of protein synthesis (Yang and Kastan, 2000). Thus a loss of ATM function in A-T patients could disrupt insulin signaling, thereby contributing to insulin resistance, glucose intolerance, and diabetes. In fact, a single nucleotide polymorphism (SNP), rs11212617 at a locus containing ATM, has been associated with glycemic response to metformin in diabetes mellitus type 2 (GoDARTs and UKPDS Diabetes Pharmacogenetics Study Group, 2010).

Mild hepatic dysfunction, as evidenced by abnormalities of serum alkaline phosphatase, serum glutamic oxalocetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and lactate dehydrogenase (LDH), is also seen in A-T (McFarlin et al., 1972). Diffuse fatty infiltration of hepatic parenchymal cells, round cell infiltration in portal regions, and parenchymal cells with nuclear swelling and vacuolation are characteristic changes. It is likely that the hepatic dysfunction is related to the generalized metabolic changes in A-T. Elevated serum levels of AFP and carcinoembryonic antigen (CEA) in A-T patients are common and thought to be indicative of some form of abnormal development in the liver (Sugimoto et al., 1978; Waldmann and McIntyre, 1972). A widespread cellular histological abnormality, nucleocytomegaly, manifested as large, bizarre, hyperchromatic nuclei (Aguilar et al., 1968; Amromin et al., 1979; Boder and Sedgwick, 1958), occurs in most organs, including the central nervous system, anterior pituitary, thyroid, adrenal glands, liver, kidney, lung, heart, and thymus, in smooth muscle cells, and in capsular cells of the spinal ganglia.

LABORATORY FINDINGS AND CELLULAR CHARACTERISTICS

CELLULAR RADIOSENSITIVITY AND CHROMOSOMAL INSTABILITY

A number of cellular and molecular features characterize A-T (Lavin, 1992) (Table 47.3). Frequent chromosome breakage, described more than four decades ago (Hecht et al., 1966; Miller, 1967), led to the classification of A-T as a chromosomal instability syndrome together with Bloom's syndrome and Fanconi's anemia (Cohen et al., 1975; Gropp and Flatz, 1967; Hecht et al., 1973; Higurashi and Conen, 1973). Clinical radiosensitivity was noted when adverse reactions in A-T patients treated with X-rays occurred (Feigin et al., 1970; Gotoff et al., 1967; Morgan et al., 1968) (Table 47.1). This adverse response was also demonstrated in A-T cells in vitro (Higurashi and Conen, 1973; Rary et al., 1975). Taylor et al. (1975) observed that A-T fibroblasts were more sensitive to ionizing radiation than control fibroblasts. These observations were confirmed and extended by others for radiation (Chen et al., 1978; Edwards and Taylor, 1981; Lehmann et al., 1982; Paterson et al., 1976), bleomycin (Cohen and Simpson, 1982; Morris et al., 1983), and treatment with neocarzinostatin (Cohen and Simpson, 1982; Shiloh et al., 1982a, 1982b, 1983).

While A-T cells are hypersensitive to ionizing radiation and radiomimetic chemicals, they have a gross repair defect in DSBs rather than in single-strand breaks. Residual DSBs can be detected in A-T cells up to 72 hours after irradiation (Table 47.3). Approximately 10 percent of breaks remain unrepaired. A number of other cellular and molecular abnormalities have been reported in A-T cells exposed to radiation (Table 47.3). A specific DNA binding protein, induced by ionizing radiation in control cells to translocate from the cytoplasm to the nucleus, was shown to be constitutively

Table 47.3 **CELLULAR AND MOLECULAR FEATURES OF A-T RELATED TO RADIATION RESPONSE**

CHARACTERISTIC	ASSAY	REFERENCE
Adverse clinical response to radiotherapy	Clinical assessment	Gotoff et al., 1967; Morgan et al., 1968; Feigin et al., 1970
Radiation-induced chromosome breaks	Cytogenetics	Higurashi and Conen, 1973; Rary et al., 1975
Cell death after irradiation	Colony survival Cell viability	Taylor et al., 1975; Chen et al., 1978
Cell death with neocarzinostatin, bleomycin (radiomimetic drugs)	Colony survival Cell viability	Cohen and Simpson, 1982; Shiloh et al., 1982a, 1982b; Morris et al., 1983
Residual DNA DSBs	Premature chromosome condensation Pulsed-field gel electrophoresis	Cornforth and Bedford, 1985; Pandita and Hittelman, 1992; Foray et al., 1997
Abnormalities in DNA topoisomerase II	DNA unwinding	Mohamed et al., 1987; Davies et al., 1989
Covalent modification of deoxyribose phosphate in DNA	HPLC analysis	Karam et al., 1990
Constitutive presence of DNA-binding protein in nucleus	Gel retardation	Singh et al., 1990; Teale et al., 1992, 1993

present in the nucleus in several A-T cell lines (Singh and Lavin, 1990; Teale et al., 1992, 1993). As discussed below, specific molecular tests, including mutation analysis of *ATM*, immunoblotting to detect the ATM protein, and specific ATM kinase assays, have all been added to laboratory tests for A-T.

CELL-CYCLE ANOMALIES

Eukaryotic cells progress through the cell cycle in an ordered series of events called G1, S (DNA synthesis), G2, and M (mitosis). Events occurring later in the cycle depend on the completion of earlier events, with control maintained through a series of checkpoints (Hoyt et al., 1991; Kastan et al., 1991; Weinert et al., 1994). Checkpoints introduce pauses to ensure the integrity of the genome by allowing for correction of episodic DNA damage or replication delays (Lukas et al., 2004). A series of yeast checkpoint null mutants have been described that lose chromosomes spontaneously and are hypersensitive to ionizing radiation (Al-Khodairy and Carr, 1992; Jimenez et al., 1992; Li and Murray, 1991; Weinert and Hartwell, 1990). These mutants fail to undergo arrest at either the G1/S or G2/M checkpoints in response to radiation damage and/or they fail to maintain the dependence of mitosis upon completion of DNA synthesis.

There are obvious parallels between the data for these yeast mutants and some of the early observations with A-T cells. The first evidence for an abnormality in cell-cycle control in A-T cells came from the observation that DNA synthesis continued after irradiation (radioresistant DNA synthesis), allowing cells to proceed through S phase unchecked (de Wit et al., 1981; Edwards and Taylor, 1981; Houldsworth and Lavin, 1980; Painter and Young, 1980). A defect in a signaling pathway from ATM through the checkpoint kinase Chk2 and Cdc25A phosphatase may be partly responsible for the unchecked cell cycle in G1/S and G2/M. Falck et al. (2001) have shown that ionizing radiation exposure normally leads to the loss of Cdc25A, preventing dephosphorylation of Cdk2 kinase and producing a transient blockade in DNA replication. This blockade is defective in A-T cells, allowing DNA replication to proceed. A delay in radiation-induced phosphorylation of replication protein A, a component of the single-strand DNA binding protein complex, has also been implicated (Liu and Weaver, 1993). Furthermore, ultraviolet (UV)-induced hyperphosphorylation of RPA (p34 subunit) depends on expression of ATM (Oakley et al., 2001).

The physiological transitory delay in DNA synthesis after irradiation also appears to be mediated through a calmodulin-dependent regulatory cascade (Mirzayans et al., 1995), and this pathway is also defective in A-T. A reduction of radiation suppression of the mitotic index in A-T fibroblasts resulted from a failure to prevent cells irradiated in G2 phase from proceeding into mitosis (Scott and Zampetti-Bosseler, 1982). This finding appeared at first to contradict evidence that A-T lymphoblastoid cells and SV40-transformed fibroblasts experienced more prolonged delay in G2/M at longer times after irradiation (Bates et al., 1985; Ford et al., 1984; Smith et al., 1985). However, it is now evident that A-T cells that are in G2 phase at the time of irradiation undergo less delay in proceeding into mitosis at short times after irradiation, but cells irradiated at other phases of the cycle proceed through to G2 phase, where they are for the most part irreversibly blocked (Beamish and Lavin, 1994). A-T cells also fail to undergo normal delay in progression from G1 to S phase after irradiation (Imray and Kidson, 1983; Nagasawa and Little, 1983). The overall picture that emerges for A-T is a failure of cell-cycle checkpoints at G1/S, during DNA synthesis, and at G2/M to respond to radiation damage (Beamish and Lavin, 1994).

An understanding of the molecular defect in A-T at the level of cell-cycle checkpoints was enabled by advances in cell-cycle control in yeast and mammalian cells (Hartwell and Smith, 1985; Lukas et al., 2004; Nurse, 1985). Passage of cells through the cycle is controlled by cyclin-dependent kinases (Pines and Hunter, 1991; Xiong et al., 1991), cyclin-kinase inhibitors (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993), other kinases and phosphatases, and a variety of other proteins, including the retinoblastoma protein and the transcription factor E2F (Hall et al., 1993; Hinds et al., 1992). The best-described disruption of this intricate pattern of control is the inhibition of DNA synthesis and slowing of cell division in response to radiation damage (Painter, 1985; Weinert and Hartwell, 1988). Kastan et al. (1991) provided an explanation for this inhibition when they demonstrated that the normal product of the tumor-suppressor gene p53 is induced by radiation and brings about delay in the passage of cells from G1 to S phase. These investigators subsequently showed that the G1/S checkpoint in A-T cells was impaired because of a defect in the p53 radiation signal transduction pathway at the level of p53 (Kastan et al., 1992). Khanna and Lavin (1993) then found that the defect in p53 induction by radiation extended to all four complementation groups of A-T, but there was normal induction of p53 in A-T cells in response to UV damage. In addition, inhibitors of protein kinase C and serine/threonine phosphatases interfered with the pathway.

Failure to observe a correlation between absence of p53 or mutated p53 and radiosensitivity (Clarke et al., 1993; Lee and Bernstein, 1993; Lowe et al., 1993; Slichenmyer et al., 1993) suggested that the ATM operates in more than one pathway affecting cell-cycle control and perhaps at other levels of cellular control. Sensitivity to ionizing radiation might be explained by a defect at the level of the G2/M checkpoint, since A-T cells accumulate at this checkpoint with time after exposure to radiation (Beamish and Lavin, 1994; Hong et al., 1994). ATM-dependent stabilization and activation of p53 are of central importance to the operation of the G1/S checkpoint in response to radiation damage to DNA. Radiation induces phosphorylation, dephosphorylation, and acetylation of p53 (Meek, 2004). Some of these modifications are ATM-dependent (Banin et al., 1998; Canman et al., 1998; Saito et al., 2002). ATM is responsible for the rapid phosphorylation of human p53 at serine 15 after irradiation, and serine 20 phosphorylation is carried out by Chk2 in an ATM-dependent manner (Chehab et al., 1999). Serine 20 phosphorylation interferes with p53's binding to its inhibitor, Mdm2, which plays a major role in its degradation. ATM also phosphorylates Mdm2 itself, to inhibit the nuclear export of the p53Mdm2

complex, thus ensuring the stabilization of p53 after irradiation (Khosravi et al., 1999; Maya et al., 2001). Thus it is evident that ATM controls G1/S checkpoint through multiple interactions.

Stabilization of p53 by radiation and other DNA-damaging agents leads to the induction of a cyclin kinase (Cdk) inhibitor, WAF1 (p21, Cip1) (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). WAF1 binds to cyclin E/cdk2 and cyclin A/cdk2 kinase complexes, and by inhibiting their activities prevents the progress of cells from G1 to S phase (Dulic et al., 1994; Waga et al., 1994). In keeping with the defective p53 response in A-T cells, Canman et al. (1994) showed a defective induction of WAF1, and the induction of Mdm2, another downstream effector of p53, was also shown to be defective (Price and Park, 1994). Khanna et al. (1995) subsequently demonstrated that the radiation signal transduction pathway operating through p53, its target gene *WAF1*, cyclin-dependent kinases, and the retinoblastoma protein (Rb) are all defective in A-T cells. Correction of the defect at the G1/S checkpoint was observed when wild-type p53 was constitutively expressed in A-T cells (Khanna et al., 1995). As expected, the WAF1 response in irradiated control cells resulted in an inhibition of cyclin-dependent kinase activity including cyclin E/cdk2, which plays an important role in the transition from G1 to S phase. No inhibition of cyclin-dependent kinase activity was observed in A-T cells, correlating with the delayed WAF1 response. An accumulation of the hypophosphorylated form of Rb protein occurred in irradiated control cells compatible with the G1/S-phase delay observed in these cells after exposure to radiation. In unirradiated A-T cells the amount of Rb protein was much higher than that in controls, and it was mainly in the hyperphosphorylated (inactive) form.

In agreement with the expected role of *ATM,* Beamish et al. (1996) demonstrated that several cyclin-dependent kinases in A-T cells are not inhibited by ionizing radiation, associated with insufficient induction of WAF1. Exposure of control lymphoblastoid cells to radiation during S and G2 caused rapid inhibition of cyclin A/cdc2 and cyclin B/cdc2 activities, respectively. Irradiation led to a 5- to 20-fold increase in cdk-associated WAF1 in these cells, accounting at least in part for the decrease in cyclin-dependent kinase activity. In contrast, radiation did not inhibit any of the cyclin-dependent kinase activities in A-T cells at short times after irradiation, nor was there any significant change in the level of cdk-associated WAF1 compared to unirradiated cells (Beamish et al., 1996). These results were similar to those reported previously for the G1 checkpoint and provided additional evidence for the involvement of ATM at multiple points in cell-cycle regulation. In relation to the abnormality in S phase, Mirzayans et al. (1995) had indirect evidence that radiation-induced cessation of DNA synthesis is mediated through a calmodulin-dependent signal transduction pathway, independent of protein kinase C (PKC) and p53; in addition to the p53 pathway, this signaling system is also defective in A-T cells.

As referred to above, A-T cells exhibit radioresistant DNA synthesis and defective S-phase checkpoint activation (Houldsworth and Lavin, 1980; Painter and Young, 1980). Falck et al. (2001) provided a functional link between ATM, the checkpoint signaling kinase Chk2, and the Cdc25A phosphatase that activates the cyclin kinase Cdk2 and control of the S-phase checkpoint. Exposure of cells to radiation leads to the activation of ATM kinase, which in turn activates Chk2 to phosphorylate Cdc25A phosphatase. This leads to the degradation of Cdc25 A, and thus Cdk2 remains phosphorylated and inactive, giving rise to a transient block in DNA replication. In cells with absent or mutated ATM, this pathway does not function, Cdc25A remains stable, and cells continue to replicate DNA in the presence of damage (radioresistant DNA synthesis).

While considerably less is known about activation of the G2/M checkpoint, maintenance of inhibitory phosphorylations on Cdc2 leads to arrest of cells in G2 (Jin et al., 1996). In response to DNA damage, Cdc25C phosphatase is phosphorylated and inactivated, again in this case preventing the dephosphorylation of Cdc2 kinase, which is required for passage of cells through G2 phase (Nilsson and Hoffmann, 2000). It is not established which kinase phosphorylates Cdc25C, but both Chk1 and Chk2 can phosphorylate this protein at serine 216 in vitro (Matsuoku et al., 1998; Sanchez et al., 1997). Phosphorylation creates a binding site for 14-3-3 protein, which results in inactivation of the phosphatase (Peng et al., 1997). Since ATM phosphorylates and activates Chk2 in response to DNA damage, it is likely that this is the pathway operating to activate the G2/M checkpoint (Blasina et el al., 1999; Brown et al., 1999).

To account for the defective radiation response in A-T cells, a "damage surveillance network" model has been proposed in which certain types of DNA damage trigger a signal transduction network with resulting activation of a group of pathways to promote genetic stability by temporarily arresting the cell cycle and enhancing DNA repair (Meyn, 1995; Meyn et al., 1994). It appears likely that ATM is recruited to sites of DSBs in DNA and subsequently signals via downstream substrates such as p53 and Chk2 to the cell-cycle machinery to delay the passage of cells between the various phases prior to completion of DNA repair. Evidence for recruitment of ATM to DNA breaks was provided by Smith et al. (1999a), who showed that ATM binds preferentially to DNA ends in both monomeric and tetrameric forms. In addition, a combination of single-strand and sheared DNA is capable of stimulating ATM kinase activity for RPA substrate (Chan et al., 2000; Gately et al., 1998). Resistance to detergent extraction of a fraction of the ATM pool after irradiation, together with colocalization of this ATM with the phosphorylated form of histone H2AX and with Nbs1 foci, suggests further that ATM associates with sites of DSBs (Andegeko et al., 2001).

THE ATM GENE AND A-T MUTATIONS

The placement of an A-T locus by Gatti et al. (1988) on chromosome 11q22–23 spurred extensive positional cloning efforts (Lange et al., 1995; McConville et al., 1994; Rotman et al., 1994; Vanagaite et al., 1995), which culminated in

identification of *ATM*, the gene mutated in A-T patients (Savitsky et al., 1995a, 1995b). *ATM* occupies 150 kb of genomic DNA and encodes a large transcript of about 13 kb representing 66 exons (Platzer et al., 1997; Savitsky et al., 1995b, 1996; Uziel et al., 1996). There is no evidence of alternative splicing within the open reading frame of the *ATM* transcript. However, extensive variability has been detected in the untranslated regions (UTRs) of the transcript, creating multiple species of ATM mRNA. Several 3′ UTRs are formed via polyadenylation of alternative sites, while at least 12 5′ UTRs are obtained via alternative splicing of the first four exons of the gene (Savitsky et al., 1997). This complex pattern suggests that synthesis of the ATM protein might be subject to posttranscriptional regulation. It has recently been shown that human microRNA, miR-421, suppresses ATM expression by targeting the 3′ UTR of *ATM* transcripts (Hu et al., 2010).

Deficiency of the ATM protein is indeed responsible for the A-T cellular phenotype. Ectopic expression of recombinant ATM protein in A-T cells complemented various features of this phenotype (Zhang et al., 1997; Ziv et al., 1997), while downregulation of ATM by means of antisense strategies conferred A-T features to various cell lines (Fan et al., 2000; Uhrhammer et al., 1999; Zhang et al., 1998). Furthermore, expression of ATM fragments containing the leucine zipper of this protein abrogated the S-phase checkpoint and increased the radiosensitivity of the human tumor cell line RKO (Morgan et al., 1997). Such protein fragments appear to act in a dominant-negative fashion, possibly by competing with ATM for specific interactor(s).

Extensive screening of the *ATM* transcript for mutations in A-T cells has been performed using a variety of methods (Angele et al., 2003; Becker-Catania et al., 2000; Broeks et al., 1998; Byrd et al., 1996; Concannon and Gatti, 1997; Fukao et al., 1998; Gilad et al., 1996a, 1996b, 1998a, 1998b; Laake et al., 1998, 2000; McConville et al., 1996; Pagani et al., 2002; Sandoval et al., 1999; Sasaki et al., 1998; Saviozzi et al., 2003; Savitsky et al., 1995a; Telatar et al., 1996; Teraoka et al., 1999; Watters et al., 1997; Wright et al., 1996). To date, over 400 separate mutations have been described. These mutations are distributed along the length of the gene, with the majority predicted to cause premature truncations (70–80 percent) and others causing small deletions, in-frame deletions, and missense changes (Hu and Gatti, 2009; Stankovic et al., 1998). Thus, *ATM* gene mutations in patients with classical A-T are expected to inactivate the ATM protein by truncating it or by deleting large segments from it. This mutation profile points to predominance of null alleles in classical A-T. Hence, mutations with milder effects on the protein might lead to non–A-T phenotypes.

Most A-T mutations are unique to single families, and the majority of patients in Europe and North America are compound heterozygotes. Interestingly, a founder effect was observed among patients of Moroccan Jewish origin, in whom one mutation is predominant (Gilad et al., 1996b). Evidence for founder mutations has also been reported for patients in Japanese (Ejima and Sasaki, 1998), Norwegian (Laake et al., 1998), and other populations (Teletar et al., 1998).

Compared to the predominance of null alleles among patients with classical A-T, a different mutation profile is emerging in "A-T variants," which show a milder phenotype, usually with later age of onset and less pronounced radiosensitivity. These patients may have leaky splicing mutations giving rise to a mixture of normal and aberrant transcripts, small in-frame deletions, or truncations of just the last few carboxy-terminal amino acids, or the defect may be in another gene (e.g., *MRE11* gene) (Dörk et al., 2004; Eng et al., 2004; Gilad et al., 1998a; McConville et al., 1996; Saviozzi et al., 2002; Stewart et al., 2001; Sutton et al., 2004; Teraoka et al., 1999).

FUNCTION OF THE ATM PROTEIN

ATM PROTEIN AND PIKK FAMILY

The open reading frame of *ATM* transcripts predicts a 370 kDa protein of 3,056 amino acids (Savitsky et al., 1995b). The ATM protein is a serine/threonine protein kinase and a member of the phosphoinositode 3-kinase-related protein kinase (PIKK) family. All members of the PIKK family are large serine/threonine protein kinases involved in signaling following cellular stress. The ATM consensus phosphorylation motif is hydrophobic-X-hydrophobic-[S/T]-Q (Derheimer and Kastan, 2010; Kim et al., 1999). The other members of the PIKK family include ATR (ATM and RAd3 related protein kinase), DNA-PKcs (DNA dependent protein kinase catalytic subunit), mTOR (mammalian target of rapamycin), and hSMG1. These members share common domain structures such as N-terminal HEAT repeats, a FAT domain, a protein kinase domain, and a C-terminal FAT-C domain (Abraham, 2004b; Derheimer and Kastan, 2010). These kinases function at the top of various signaling cascades that sense specific types of stresses or stimuli related to cellular growth and are critical for the activation of cellular responses to these stimuli. Most of the PIKK family members are involved in sensing and responding to DNA damage and in maintaining genomic stability (Table 47.4). PIKK family members, as another common feature, associate with proteins or protein complexes that facilitate their activation and function. ATM binds to the MRN (Mre11, Rad50, Nbs1) heterotrimer at DSB sites. This binding places the ATM on chromatin, to efficiently activate the downstream signaling and DNA repair mechanisms (Falck et al., 2005; Lovejoy and Cortez, 2009).

The ATM ortholog in the budding yeast *Saccharomyces cerevisiae* is Tel1p, whose ectopic expression in human A-T cells partially complements their phenotype (Fritz et al., 2000). Tel1p and another PIKK member in the budding yeast, Mec 1p, closely collaborate in maintaining telomere length and mediating cellular responses to DNA damage. Tel1p, which has a protein kinase activity (Mallory and Petes, 2000), has been studied mainly with regard to its role in maintaining telomere length (Greenwell et al., 1995; Ma and Greider, 2009; Morrow et al., 1995; Ritchie et al., 1999; Seidel et al., 2008). *TEL1* mutations lead to shortened telomeres, a typical characteristic of human A-T cells. Interestingly, in this function, Tel1p cooperates with the double-strand breakage repair

Table 47.4 ATM-RELATED PROTEINS CONTAINING A PI3-KINASE–LIKE DOMAIN

PROTEIN	ORGANISM	SIZE (A.A.)	MUTANT PHENOTYPE	PREDICTED FUNCTION (ENZYMATIC ACTIVITY)
Te11p	Budding yeast	2789	Teleomere shortening	Regulation of telomere length
Meclp	Budding yeast	2368	Sensitivity to DNA-damaging agents; mitotic instability; defects in meiotic recombination and cell cycle checkpoints	Regulates cell-cycle checkpoints responding to DNA damage
Rad3p	Fission yeast	2386	Sensitivity of DNA-damaging agents; mitotic instability; defective DNA repair and cell cycle checkpoints	Regulates cell-cycle checkpoints responding to DNA damage
Mei-41	Fruit fly	2356	Sensitivity to DNA-damaging agents; mitotic instability; defective DNA repair and cell cycle checkpoints	Regulates cell-cycle checkpoints responding to DNA damage
Tor1p	Budding yeast	2470	Rapamycin resistance; perturbation of G1 cell-cycle progression	Binds rapamycin, FKBP12; Heitman et al., 1991 involved in G1/S transition
Tor2p	Budding yeast	2473	Rapamycin resistance; perturbation of G1 cell-cycle progression	Binds rapamycin, FKBP12; involved in G1/S transition
MTOR (FRAP)	Mammalian	2549	Rapamycin resistance; perturbation of G1 cell-cycle progression	Binds rapamycin, FKBP12
RAFT1	Mammalian	2550		Involved in G1/S transition; activates $p70^{S6}$ kinase (autophosphorylation on Ser)
FRP1/ATR	Mammalian	2644	Embryonic lethal in mice; no known human syndrome	Protein kinase responding to UV, hydroxyurea
DNA-PKcs	Humans	4096	SCID phenotype in mice; radiation sensitivity; immunodeficiency; defective repair of double-strand breaks and V(D)J recombination	Signals presence of DNA damage to cellular regulatory systems (Ser/Thr protein kinase)
TRAPP	Mammalian	3828	DNA repair; recruits histone acelylases, oncogene activation	No kinase activity

complex Mre11/Rad50p/Xrs (Ritchie and Petes, 2000), whereas in human cells, ATM functionally interacts with the orthologous protein complex in the DNA damage response. The synergism between Tel1p and Mec1p in both telomere maintenance and cellular responses to DNA damage has been clearly documented in double-mutant *tel1/mec1* yeast, which are extremely sensitive to DNA damage and senesce prematurely (Greenwell et al., 1995; Ritchie et al., 1999; Vialard et al., 1998). While *tel1* mutants are not hypersensitive to DNA damaging agents, an extra copy of *TEL1* can largely complement such sensitivity in *mec1* mutants (Morrow et al., 1995).

The closest human ATM homolog is the ATR protein, which represents a separate branch of the PIKK family. ATR is a protein kinase that shares many substrates with ATM and acts in partial redundancy with ATM in a number of damage response pathways (Shechter et al., 2004). ATR and ATR-interacting protein (ATRIP) control S-phase progression in response to DNA damage and replication fork stalling, ultimately resulting in the downstream inhibition of the S-phase kinases that function to initiate DNA replication at origins of replication (Shechter et al., 2004). Disruption of the *ATR* gene in mice leads to early embryonic lethality before embryonic day 8.5 (Brown and Baltimore, 2000; de Klein et al., 2000). A hypomorphic mutation (A2101G) in the human *ATR* gene causes a rare form of Seckel syndrome (O'Driscoll et al., 2003) designated ATR-Seckel (Alderton et al., 2004; O'Driscoll et al., 2004). Genetically and clinically, Seckel syndrome is a heterogeneous disorder characterized by severe intrauterine growth retardation, proportionate dwarfism, and microcephaly, with skeletal and brain abnormalities (OMIM #210600) (reviewed by O'Driscoll et al., 2004). Although lymphoma has been reported in some Seckel patients, they tend not to have ataxia or immunodeficiency. Patients with Seckel syndrome display features including microcephaly and dysmorphic facies that are commonly found in other syndromes associated with impaired responses to DNA damage. Alderton et al. (2004) found that ATR-Seckel cells exhibit a range of aberrant responses to agents that cause replication stalling. They also exhibit supernumerary centrosomes in mitotic cells, demonstrating a novel role for ATR in the maintenance of centrosome stability.

Overexpression of a "kinase-dead" form of ATR gives rise to a dominant-negative effect leading to increased sensitization to DNA damaging agents and defective cell-cycle checkpoint activation (Cliby et al., 1998; Wright et al., 1998). ATR-deficient cells are hypersensitive to UV and hydroxyurea (HU), in contrast to A-T cells, in which γ-radiation causes hypersensitivity. Indeed, the phosphorylation of Rad17 induced by UV and HU is dependent on ATR, whereas that

induced by γ-rays is dependent on ATM (Bao et al., 2001). ATR appears to lie directly upstream from the checkpoint protein Chk1, which is activated in response to DNA replication blockage and agents that cause genotoxic stress to delay cells at the G2 checkpoint (Zhao and Piwnica-Worms, 2001).

In contrast to ATM, whose intrinsic kinase activity is contained in a single protein molecule, ATR is constitutively complexed and dependent on the accessory protein, ATRIP (Cortez et al., 2001). ATRIP is phosphorylated by ATR, regulates ATR expression, and is essential for proper ATR activity. ATR and ATRIP both localize to intranuclear foci after DNA damage or inhibition of replication. Small, interfering RNA directed against ATRIP causes the loss of both ATRIP and ATR expression and the loss of checkpoint responses to DNA damage, indicating the mutual dependence of these two proteins (Cortez et al., 2001). Replication protein A (RPA), a protein complex that associates with single-stranded DNA (ssDNA), is required for the recruitment of ATR to sites of DNA damage and for ATR-mediated Chk1 activation in human cells. In vitro, RPA stimulates the binding of ATRIP to ssDNA. The binding of ATRIP to RPA-coated ssDNA enables the ATR/ATRIP complex to associate with DNA and stimulates phosphorylation of the Rad17 protein that is bound to DNA (Zou and Elledge, 2003). In fact, ATM and ATR are involved together in phosphorylation of many cell-cycle checkpoint and DNA repair proteins (Lavin and Kozlov, 2007; Matsuoka et al., 2007). Encompassing 700 proteins, more than 900 regulated phosphorylation sites that contain a consensus ATM and ATR phosphorylation motif (S/T-Q) have been identified by a large-scale proteomic analysis (Matsuoka et al., 2007).

ATR orthologs are typically involved in maintaining genome stability and responding to DNA damage. The yeast ATR orthologs, Mec1p in the budding yeast and Rad3p in the fission yeast, maintain genomic stability and activate DNA damage responses by phosphorylating key proteins in these pathways (reviewed in Zhou and Elledge, 2000; Humphrey, 2000). As mentioned above, Mec1p collaborates with the ATM ortholog, Tel1p, in the damage response pathway and telomere maintenance in *S. cerevisiae* (Longhese et al., 2000; Neecke et al., 1999). Notably, Mec1p-dependent processes include cell-cycle checkpoints induced by DNA damage or replication arrest (Clarke et al., 1999; Santocanale and Diffley, 1998; Vallen and Cross, 1999); redistribution of the Sir3 silencing protein from telomeres to DSBs (Mills et al., 1999); meiotic recombination (Grushcow et al., 1999); and silencing of gene expression at telomeres (Craven and Petes, 2000). The Rad3p of *Schizosaccharomyces pombe* is critical for the immediate activation of cell-cycle checkpoints following DNA damage and for the activation and maintenance of responses to replication arrest. The mechanisms in each response are different and carried out by different effectors (Martinho et al., 1998). Many of the relevant pathways are conserved through evolution; hence, in mammalian cells, ATM and ATR phosphorylate orthologs of Mec1p and Rad3p substrates. In *Drosophila melanogaster* mutants in the ATR ortholog *mei-41* exhibit chromosomal instability, radiation sensitivity, and defective activation of cell-cycle checkpoints by ionizing radiation (Hari et al., 1995). Recombination-defective lines of flies reveal a role for Mei-41 protein in meiotic precocious anaphase in females (McKim et al., 2000).

The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) is another major member of the PIKK family (reviewed by Burma and Chen, 2004). DNA-PKcs is recruited to DSB sites by the Ku70/Ku80 heterodimer and appears to play a central role in the rejoining process, in V(D)J recombination, and in triggering apoptosis in response to severe DNA damage or critically shortened telomeres. DNA-PK also appears to be involved in mounting an innate immune response to bacterial DNA and to viral infection. Because DNA-PK localizes very rapidly to DNA breaks and phosphorylates itself and other damage-responsive proteins, it appears that DNA-PK serves as both a sensor and a transducer of DNA-damage signals. ATM- and ATR-dependent pathways appear to be independent of DNA-PK (Araki et al., 1999; Khosravi et al., 1999). However, the critical role of DNA-PK in DSB response is underscored by the *scid* phenotype of DNA-PK–deficient mice, which includes radiosensitivity, chromosomal instability, immunodeficiency, and cancer predisposition (Jhappan et al., 1997).

The TOR subfamily of the PIKKs represents a group of proteins whose role is to convey, via their kinase activity, extracellular signals rather than dealing with DNA damage (Proud, 2004). The TOR proteins signal nutrient availability (e.g., amino-acid levels) and pass on certain mitogenic stimuli to the mRNA translation machinery. One known mechanism is TOR-dependent phosphorylation of the $p70^{S6k}$ kinase. The pathways controlled by the mammalian homolog in this group, mTOR/FRAP/RAFT, may end up regulating both protein translation and transcription of rRNA and tRNA. Importantly, TOR-mediated pathways appear to intersect with certain P13K pathways.

A more recent addition to the PIKK family is the hSMG1 protein (Abraham, 2004a). The protein kinase activity of hSMG-1 resembles that of ATM, both in terms of substrate specificity and its sensitivity to inhibition by the fungal metabolite wortmannin. hSMG-1 is the ortholog of a *Caenorhabditis elegans* protein, CeSMG-1, which has been genetically linked to a critical mRNA surveillance pathway termed *nonsense-mediated decay* (NMD). The function of NMD is to mark for rapid degradation mRNAs that bear a premature termination codon. hSMG-1 is also a central player in the NMD pathway in human cells. In addition, hSMG-1, like ATM, appears to be involved in the recognition and/or repair of damaged DNA in these cells.

Finally, members of the TRRAP (transformation/transcription domain-associated protein) branch of the PIKK family are devoid of kinase activity because of inactivating sequence alterations in their kinase domains. The mammalian (TRRAP/PAF400) and yeast (Tra1p) homologs are part of large protein complexes involved in transcriptional regulation and chromatin remodeling via their histone actetyltransferase activity (Grant et al., 1998; Saleh et al., 1998; Vassilev et al., 1998). TRRAP/PAF400 is a co-activator of the c-Myc and E2F transcription factors and recruits the histone acetyltransferase GCN5 to c-Myc (McMahon et al., 1998, 2000).

The existence of the TRRAP group in the PIKK family raises the possibility that members residing in other branches of the PIK-related kinase phylogenetic tree are involved in gene regulation and shaping of chromatin. In general, despite the apparently diverse functions of the different branches of this important protein family, they may share more functions than meet the eye.

ATM FUNCTIONS

ATM Activation

ATM is a predominantly nuclear protein with a strong serine/threonine kinase activity. Similar to DNA-PK and ATR, ATM's kinase activity appears to be directed at serine or threonine residues that are immediately followed by a glutamine (Kim et al., 1999; O'Neil et al., 2000). ATM plays a crucial role in the early response to the induction of DSBs in the DNA (Kurz and Lees-Miller, 2004; Shiloh, 2003). Immediately after the formation of DSBs in the DNA, ATM's kinase activity is enhanced (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998). The activated ATM then phosphorylates an extensive array of target proteins, each of which is a key player in a specific damage response pathway (Fig. 47.1). Bakkenist and Kastan (2003) discovered that ATM molecules are inactive in undamaged cells, being held as dimers or higher-order multimers. In this configuration, the kinase domain of each molecule is blocked by the *FAT domain* of the other. Following DNA damage, each ATM molecule phosphorylates the other on a serine residue at position 1981 within the FAT domain, an autophosphorylation that releases the two molecules from each other's grip, turning them into fully active monomers. Additional phosphorylation sites of Ser(367), Ser(1893), Thr(P)(1885), and Ser(P)(2996) (Kozlov et al., 2006, 2011) and other post-translational modifications also play a role in ATM activation. The ubiquitously expressed histone acetyl-transferase, TIP60, acetylates ATM on Lysine 3016, along with autophosphorylation (Sun et al., 2010) to activate ATM. Within minutes after the infliction of as few as several DSBs per genome, most ATM molecules become vigorously active. Another immediate step in ATM activation following DNA damage is rapid adherence of a portion of activated ATM to DSB sites (Andegeko et al., 2001). Both chromatin-bound ATM and free ATM are autophosphorylated on serine 1981 (Uziel et al., 2003). Therefore, following DSB induction, activated ATM seems to divide between two fractions: one is chromatin bound and the other is free to move throughout the nucleus. Importantly, many ATM substrates are phosphorylated by the chromatin-bound fraction of ATM, at the damaged sites (Lukas et al., 2003).

One of the first processes initiated by DSBs is the massive phosphorylation of the tail of a histone protein variant H2AX (Fernandez-Capetillo et al., 2004). H2AX phosphorylation is ATM-dependent following DSB induction (Burma et al., 2001) and ATR-dependent following replicative stress (Ward and Chen, 2001). Foci of γH2AX, visualized by immunofluorescence as a marker for DNA breaks (Rogakou et al., 1999), are rapidly formed at the DSB sites and are essential for further recruitment of repair factors such as the Mre11/Rad50/Nbs1 (MRN) complex, Rad51, and Brca1 (Celeste et al., 2002). Disappearance of γH2AX foci, probably representing the restoration of the local chromatin environment, correlate with completed DNA repair (Derheimer and Kastan, 2010; Kinner et al., 2008). A-T cells show persistent γH2AX foci following ionizing radiation (Riballo et al., 2004). For efficient DNA repair, the surrounding heterochromatin structure needs to decondense and relax to provide access to the damaged DNA. This relaxation is somewhat achieved by ATM via phosphorylating and inhibiting the transcriptional repressor KAP1 (KRAB-associated protein 1), a core component of heterochromatin (Cann and Dellaire, 2011; Derheimer and Kastan, 2010; Goodarzi et al., 2008; Ziv et al., 2006).

The Mre11/rad50/Nbs1 (MRN) complex serves as a sensor of DSBs caused by DNA-damaging agents (Stracker et al., 2004). It is involved in the initial processing of such DSBs by virtue of the nuclease activity of one of its components—the Mre11 protein. MRN is also involved in processing DSBs that are formed during meiotic recombination and in telomere maintenance. The Nbs1 protein is a direct substrate of ATM but also seems to serve as an adaptor in the phosphorylation of other ATM substrates such as Chk2 (Buscemi et al., 2001; Girard et al., 2002) and Smc1 (Kim et al., 2002b; Yazdi et al., 2002), particularly at low damage levels. Similarly, the Brca1 protein, itself an ATM substrate, is an adaptor in the phosphorylation of other ATM substrates (Foray et al., 2003). Therefore, phosphorylation of one substrate is required for the phosphorylation of others. A molecular explanation for the adaptor role of the MRN complex was provided by Lee and Paull (2004), who showed that MRN facilitates in vitro the binding between ATM and some of its substrates. MRN also plays a role in the actual activation of ATM. The presence of a functional MRN complex is required for full activation of ATM, particularly at low damage levels (Carson et al., 2003; Horejsi et al., 2004; Uziel et al., 2003) and for recruitment of ATM to the damaged sites (Kitagawa et al., 2004). A molecular explanation for these observations was provided by Costanzo et al. (2004), who showed that in *Xenopus* extracts, ATM, MRN, and broken DNA fragments assemble into an "activation complex" whose formation is required for full activation of ATM.

Another protein that appears to facilitate the phosphorylation of certain ATM substrates is p53 binding protein 1 (53BP1) (Mochan et al., 2004). Like Nbs1, 53BP1 contains a BRCT (breast cancer C-terminus) domain, is recruited to DSB-induced nuclear foci, is required for proper activation of certain cell-cycle checkpoints, and is itself an ATM substrate (DiTullio et al., 2002; Fernandez-Capetillo et al., 2002; Wang et al., 2002). Another BRCT-containing protein phosphorylated by ATM is MDC1/NFBD1 (Stucki and Jackson, 2004). MDC1, bound to γH2AX and phosphorylated by ATM, serves as a docking station for DNA-repair proteins. Ring-finger ubiquitin ligases RNF8 and RNF168 are recruited to the DSB site, followed by ubiquitination of γH2AX and maintenance of 53BP1 and BRCA1, and ATM at the DSB site (Derheimer and Kastan, 2010; Lavin, 2008).

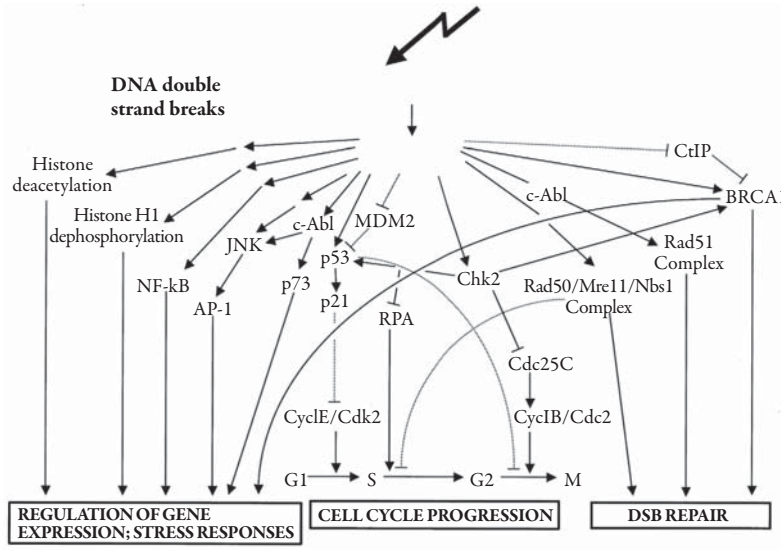

Figure 47.1 Scheme of ATM-dependent pathways in the double-strand break (DSB) response network, highlighting ATM effectors. Arrows indicate stimulation, while T-shaped lines represent inhibition. The transition from inactive ATM homodimers into active monomers and the role of the Rad50/Mre11/Nbs1 (MRN) complex upstream of this process are indicated. ATR phosphorylates at least some of ATM's substrates in response to these DNA lesions at later time points and slower kinetics. (Reproduced from Shiloh et al., 2004, with permission.)

ATM, apart from being activated by DSBs as described above, can be directly activated by oxidative stress in the absence of MRN complex (Guo et al., 2010). Thus, ATM appears to act as a redox sensor and may be regulating global cellular responses to oxidative stress. In fact, A-T cells have impaired mitochondrial function and increased levels of reactive oxygen species (ROS) as well as mitochondrial DNA deletions in the region that encodes cytochrome *c* oxidase (COX) (Lavin, 2008; Patel et al., 2011). More recently, Valentin-Vega et al., 2012 have shown that ATM-deficient thymocytes in mice have significant reductions in mitochondrial electron transport chain activity, decreases in intracellular ATP levels, altered mitochondrial ultrastructural organization, and enhanced mitochondrial ROS in the presence of increased mitochondrial numbers possibly due to decreased mitophagy. Therefore, the authors have suggested that ATM plays direct roles in modulating mitochondrial homeostasis and A-T should be considered partly a mitochondrial disease.

ATM SUBSTRATES AND DOWNSTREAM PATHWAYS

ATM has multiple substrates. Proteomic analyses of proteins phosphorylated in response to DNA damage on consensus sites recognized by ATM and ATR suggest as many as 700 substrates (Linding et al., 2007; Matsuoka et al., 2007). The high number of ATM targets suggests that ATM plays a role in many cellular responses and signaling pathways yet to be discovered. These proteomic analyses also draw attention to the cytoplasmic ATM in peroxisomes, endosomes, or in soluble form, as a protein with a more general role in cellular pathways such as in insulin signaling (Guo et al., 2010; Lavin, 2008; Patel et al., 2011; Yang et al., 2011; Yang and Kastan, 2000).

ATM orchestrates a remarkably broad cellular response to DSBs. A simplified network of ATM-mediated pathways (Fig. 47.1) uses several sophisticated strategies. The first strategy is to approach the same effector from several different directions. A prime example is the G1/S checkpoint (reviewed by Shiloh, 2003; Kurz and Lees-Miller, 2004). A main component in this cell-cycle checkpoint is activation and stabilization of p53, which in turn activates transcription of the gene that encodes the CDK2-cyclin-E inhibitor WAF1 (also known as p21 and CIP1) (Meek, 2004; Oren, 2003). p53 is phosphorylated by ATM on Ser15 (Banin et al., 1998; Canman et al., 1998). This contributes primarily to enhancing p53's activity as a transcription factor (Ashcroft et al., 1999; Dumaz and Meek, 1999; Khosravi et al., 1999). ATM also phosphorylates and activates Chk2 (Ahn et al., 2004), which phosphorylates p53 on Ser20. This process interferes with the p53–MDM2 interaction. The oncogenic protein Mdm2 is both a direct and indirect inhibitor of p53, as it serves as a ubiquitin ligase in p53 ubiquitylation, which mediates its proteasome-mediated degradation (Alarcon-Vargas and Ronai, 2002; Brooks and Gu, 2004). ATM also directly phosphorylates Mdm2 on Ser395, which interferes with nuclear export of the p53Mdm2 complex and hence the degradation of p53 (Khosravi et al., 1999; Maya et al., 2001). Phosphorylations of p53 on Ser9 and Ser46 (Saito et al., 2002) and dephosphorylation of Ser376 (Waterman et al., 1998) are ATM-dependent as well, although the function of these changes is unclear.

This series of ATM-dependent modifications that activate and stabilize p53, although perhaps not complete, illustrates the elaborate way in which ATM handles a single effector. This principle is also seen in ATM-mediated activation of the Brca1 tumor-suppressor protein following DNA damage. Brca1 is involved in the early stage of the DNA-damage response as well as in activating downstream pathways (Ting and Lee, 2004). Brca1 was found to be associated

with large protein complexes that contain DSB repair and mismatch repair enzymes as well as ATM (Wang et al., 2000b). It also activates the expression of certain damage-responsive genes and is involved in the S-phase and G2/M checkpoints (El-Deiry, 2002; Jasin, 2002; Venkitaraman, 2002; Yarden et al., 2002). ATM phosphorylates Brca1 on several sites (Cortez et al., 1999; Gatei et al., 2000a, 2001b). Importantly, whereas ATM-mediated phosphorylation of Brca1 on Ser1387 activates Brca1 as a regulator of the intra–S-phase checkpoint (Xu et al., 2002), its phosphorylation by ATM on Ser1423 spurs its involvement in the G2/M checkpoint (Xu et al., 2001). Thus, phosphorylation on different sites may direct an effector to act in different pathways. The Chk2 kinase, which is activated by ATM, adds yet another phosphorylation on the Brca1 molecule (Lee et al., 2000), while at the same time ATM phosphorylates CtIP, an inhibitor of Brca1, on two residues, inhibiting its function and further stimulating Brca1 (Li et al., 2000). ATM's hold on downstream pathways is demonstrated by not only its multipronged grip of specific pathways but also its ability to approach the same endpoint from several different directions. A notable example is, again, the intra-S checkpoint: several ATM-controlled pathways converge to regulate this crucial cellular response to DSBs (Fig. 47.1). At least five ATM-mediated pathways seem to be involved in this checkpoint. In addition to Brca1, ATM phosphorylates Nbs1 (Gatei et al., 2000b; Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000), a component of the multifunctional Mre11Rad50Nbs1 (MRN) complex involved in DSB sensing (Stracker et al., 2004). Of several ATM phosphorylation sites on Nbs1, Ser343 and Ser278 seem to be particularly important for its unknown role in this checkpoint (Lim et al., 2000; Zhao et al., 2000). Another ATM substrate in the intra-S checkpoint pathway is the Smc1 (structural maintenance of chromosomes 1) protein. This protein, known primarily for its involvement in sister chromatid cohesion, is phosphorylated by ATM on two serine residues; interference with this phosphorylation abrogates the S-phase checkpoint (Kim et al., 2002b; Yazdi et al., 2002). Importantly, cells from mice in which ATM phosphorylation sites on Smc1 had been abolished had an S-phase checkpoint defect, decreased survival, and increased chromosomal aberrations after DNA damage (Kitagawa et al., 2004). A recently identified effector of ATM in the intra-S checkpoint is the FancD2 protein, which is phosphorylated by ATM on Ser222 following DSB induction and undergoes Brca1-mediated mono-ubiquitylation (Taniguchi et al., 2002). FancD2 is a member of a multiprotein complex, defects of which lead to another genomic instability syndrome, Fanconi's anemia. At the same time, Chk2 and another ATM/ATR-activated kinase, Chk1, take care of several checkpoints. Both of them phosphorylate the checkpoint phosphatase Cdc25A, which marks it for degradation (Falck et al., 2001, 2002; Mailand et al., 2000, 2002; Zhao et al., 2002). Cdc25A's duty is to dephosphorylate—and hence maintain the activity of—the cyclin-dependent kinases Cdk2 and Cdk1. Cdk2 drives both the G1/S transition and S phase, and Cdk1 mobilizes the G2 phase onto mitosis. Therefore, the destruction of Cdc25A contributes to the G1/S, intra-S, and G2/M checkpoints (Fig. 47.1). The Cdc25A-mediated component of the G1/S checkpoint is rapid and, unlike the p53-mediated component, is not dependent on gene activation and protein synthesis (Falck et al., 2002).

Notably, in addition to ATM's versatility as a protein kinase with numerous substrates, the ATM web contains protein kinases that are themselves capable of targeting several downstream effectors simultaneously, and so concomitantly control subsets of pathways. Chk2 is known to phosphorylate p53, BrcA1, Cdc25A, and Cdc25C (Bartek et al., 2001; McGowan, 2002) (Fig. 47.1). The Chk2-Cdc25C pathway is similar to the previously mentioned Chk2-Cdc25A pathway, but may act instead at the G2/M transition. Here, phosphorylation of Cdc25C by activated Chk2 is thought to lead to cytoplasmic sequestration of Cdc25C, which prevents activation of Cdk1.

Most of these pathways have not been completely characterized, and the involvement of ATM substrates in them has been inferred from defective activation of specific checkpoints following abrogation of ATM-mediated phosphorylation of these proteins. An interesting pathway is mediated by phosphorylation of hRad17, the human ortholog of Rad17 from the fission yeast *Schizosaccharomyces pombe*. hRad17 is phosphorylated in an ATM/ATR-mediated manner on two serine residues, and this phosphorylation is important for proper G1/S and G2/M checkpoint functions (Bao et al., 2001; Post et al., 2001). hRad17 is involved in a very early event in the DSB response: it loads a trimolecular complex onto the DNA that consists of the human orthologs of the fission yeast proteins Rad9, Hus1, and Rad1 ("the 9-1-1 complex"). This complex acts as a sliding clamp, probably a damage sensor (Parrilla-Castellar et al., 2004). Although this process may be upstream of the transducer recruitment, two essential proteins in this process, hRad17 and hRad9, are also phosphorylated in an ATM/ATR-dependent manner and participate in downstream checkpoint pathways (Bao et al., 2001; Chen et al., 2001; Post et al., 2001).

ATM AND GENE TRANSCRIPTION

Many damage responses modulate expression of genes. Indeed, a major ATM target, p53, is a transcription factor. Another transcription factor with a central role in cell-cycle control, E2F1, has been reported to be phosphorylated and stabilized in an ATM-dependent manner (Lin et al., 2001). In addition, it is becoming evident that stress-response pathways that are best known for responding to other triggers in an ATM-independent manner, such as those mediated by the mitogen-activated protein kinases or the transcription factor NF-KB, also contain a DNA damage-responsive component (Pearce and Humphrey, 2001). Interestingly, when these pathways are activated by DSBs, their response becomes ATM-dependent (Bar-Shira et al., 2002; Li et al., 2001; Wang et al., 2000a). The direct targets of ATM in these pathways have not been elucidated, but these observations expand the ATM-dependent network considerably.

ATM AND DAMAGE-INDEPENDENT GENOMIC STABILITY

ATM helps maintain genomic stability through mechanisms other than responding to DSBs caused by damaging agents. T cells from A-T patients have abnormally shortened telomeres, abnormal association of chromosome ends, telomere clustering, and altered interaction between the telomeres and the nuclear matrix (Pandita, 2001). ATM is functionally linked to telomere maintenance, a process crucial to aging and cancer (Chan and Blackburn, 2002; Kim et al., 2002a; Maser and DePinho, 2002; Misri et al., 2008; Pandita, 2001). Here, ATM may be responding continuously to an ongoing process rather than abruptly and vigorously to an acute insult (Karlseder et al., 1999). The functional relationships between ATM and the telomere maintenance machinery have been illuminated by a study that generated mice doubly null for Atm and the telomerase RNA component Terc (Wong et al., 2003). These animals showed increased genomic instability, enhanced aging, and premature death, with a general proliferation defect extending into stem- and progenitor-cell compartments. Interestingly, the rate of T-cell malignancies in these mice was reduced rather than enhanced.

Free ends of the chromosome are masked by a protein complex, Shelterin, so that they are not recognized as free ends of a DSB (Palm and de Lange, 2008). Multiple proteins, including TIN2, TRF1, TRF2, TPP1, POT1, and RAP1, compose this telomeric protection complex. TRF1 and TRF2 are substrates of ATM (Derheimer and Kastan, 2010; Tanaka et al., 2005). Phosphorylation of TRF1 seems to suppress TRF1-mediated apoptosis following DNA damage (Kishi et al., 2001). Inhibition of TRF1 in A-T cells rescues telomere shortening and decreases the radiosensitivity of these cells, providing further evidence of the link between telomere metabolism and the DNA damage response (Kishi and Lu, 2002). Inhibition of TRF2 causes disruption of the Shelterin complex, thereby exposing the free telomeric ends to be recognized as DSBs, and activation of an ATM-dependent DNA damage response that would result in telomere dysfunction (Derheimer and Kastan, 2010; Palm and de Lange, 2008).

Further evidence of ongoing activity of ATM at sites of normally occurring breakage and reunion of DNA was provided by its presence at sites of V(D)J recombination (Bredemeyer et al., 2008; Kracker and Durandy, 2011; Perkins et al., 2002). At those sites, p53 phosphorylated at the ATM target residue was also present, indicating that ATM continuously surveys the V(D)J recombination process and can induce a damage response if DSBs are not sealed in a timely manner. Indeed, A-T patients often show clonal translocations involving the sites of the T-cell receptor and immunoglobulin genes, demonstrating the consequences of absence of this surveillance. These translocations often herald the onset of lymphoid malignancy (Xu, 1999).

ATM-DEFICIENT MICE: AN ANIMAL MODEL OF A-T

Mouse models of A-T were established in several laboratories by forming null alleles of the mouse homolog, *Atm* (Barlow et al., 1996; Elson et al., 1996; Herzog et al., 1998; Xu et al., 1996; Borghesani et al., 2000). $Atm^{-/-}$ mice share many clinical features of human A-T and highlight several features that have not received sufficient attention in humans and might be essential to understanding the functions of ATM. The reduced number and abnormal function of B and T cells, radiosensitivity, and a strong predisposition to cancer are similar between humans and mice. However, $Atm^{-/-}$ animals die almost exclusively from thymic lymphomas, whereas human patients develop leukemias, lymphomas, and solid tumors. Growth retardation is much more pronounced in $Atm^{-/-}$ mice than in human A-T patients. Fibroblasts from $Atm^{-/-}$ mice grow slowly and exhibit inefficient progression from G1 to S phase following serum stimulation (Xu and Baltimore, 1996). Atm-deficient mouse fibroblasts manifest radiosensitivity, genome instability, and defective radiation-induced cell-cycle checkpoints. An *Atm* knock-in mutant mouse harbors a 9 nucleotide in-frame deletion (7666del9) corresponding to a common human mutation (7636del9) and producing near-full-length Atm protein (Spring et al., 2001). The overall phenotype is similar to $Atm^{-/-}$ mice except for longer lifespan in nonspecific pathogen-free conditions, a lower incidence of thymic lymphomas, and more varied tumor types in older animals.

The neurological deficit in $Atm^{-/-}$ mice is much less pronounced than in humans and can be elicited only by special testing. Moreover, the cerebella of these animals appear normal upon histopathological examination. The use of electron microscopy has provided evidence for degeneration of several different neuronal cell types in the cerebral cortex in 25 percent of neuronal profiles of 2-month-old $Atm^{-/-}$ mice (Kuljis et al., 1997). Degenerating Purkinje cells had abnormalities in nucleoplasm and cytoplasm as well as crenated cell membranes from which filiform appendages originated. Degeneration was also observed in other types of neurons and glial cells. Replacement of the Rad3 homology domain of Atm with a *neoIr* gene generated an $Atm^{-/-}$ mouse with ectopic and abnormally differentiated Purkinje cells (Borghesani et al., 2000). Although gross neurological abnormalities were found, none of the other $Atm^{-/-}$ mice exhibited such changes. However, $Atm^{-/-}$ mice show age-dependent defects in Ca^{2+} spike bursts and calcium currents (Chiesa et al., 2000), and cerebellar Purkinje cells from $Atm^{-/-}$ animals are in a state of oxidative stress (Barlow et al., 1999; Kamsler et al., 2001; Watters et al., 1999).

Male and female sterility due to absence of mature gametes is another striking feature that has not received special attention in humans. Atm is clearly essential for mouse germ-cell development. Xu et al. (1996) noticed that meiosis is arrested in these mice at the zygotene/pachytene stage of the first meiotic prophase as a result of abnormal chromosomal synapsis and subsequent fragmentation. Using electron microscopy, Barlow et al. (1998) showed that male and female gametogenesis is disrupted in $Atm^{-/-}$ mice as early as leptonema of prophase 1, resulting in apoptotic degeneration. These results are corroborated by the observation that Atm is physically associated with synapsed chromosomal axes at meiosis (Keegan et al., 1996; Plug et al., 1997). These findings provide direct evidence of the role of Atm in

recognizing specific DNA structures formed during meiotic recombination.

In summary, observations in mouse knockout models highlight the role of Atm in normal somatic cell growth and chromosomal recombination in meiosis, in addition to its recognized function in radiation-induced signal transduction. Further genetic manipulation of $Atm^{-/-}$ mice will enable the elucidation of additional features of A-T previously undisclosed in the human disease (Barlow et al., 1996; Elson et al., 1996; Xu and Baltimore, 1996; Xu et al., 1996).

DIAGNOSIS OF A-T

As in all primary immunodeficiency disorders, diagnosis of A-T starts with a high index of suspicion. In the absence of family history, the disease is usually detected by a pediatric neurologist on the basis of anomalies in station and gait accompanied by telangiectasias. However, telangiectasias usually appear later than ataxia, between 2 and 8 years of age. Before then, differential diagnosis of ataxia is crucial. Ataxia may be a part of another syndrome such as Friedreich's ataxia, which typically has a later onset with pes cavus and kyphoscoliosis, unusual manifestations in A-T. Ataxia may also be due to a cerebral neoplasm or hematoma, infectious encephalitis, postinfectious encephalomyelitis, progressive rubella panencephalitis, or subacute sclerosing panencephalitis. A number of metabolic diseases of infancy and childhood, including Gaucher disease, Niemann-Pick disease, GM1 and GM2 gangliosidoses, metachromatic leukodystrophy, or Krabbe leukodystrophy, may manifest with ataxia. A thorough medical history and physical examination allows discrimination between A-T and two other metabolic disorders, Hartnup disease and maple syrup urine disease; in both of these, ataxia is episodic rather than progressive as in A-T. Laboratory assays are quite helpful in diagnosing metabolic diseases.

A-T can also be readily distinguished from the hereditary motor-sensory neuropathies Charcot-Marie-Tooth disease (distal weakness, pes cavus, hyporeflexia, and mild to moderate sensory loss), type III disease (distal weakness, nerve hypertrophy, and electromyographic abnormalities), and Refsum disease (polyneuropathy and ataxia). Other syndromes exhibiting ataxia include Machado-Joseph disease, dentatorubral degeneration of Ramsay Hunt, and olivopontocerebellar atrophy. Each of these differs from A-T by having a different pattern of inheritance or age of onset.

A number of laboratory tests have been used to support the clinical diagnosis of A-T (Table 47.5) since the description of the disease. Reduced or absent IgA and elevated AFP levels are nonspecific but supportive of the clinical diagnosis. The demonstration of frequent chromosome breakages (Hecht et al., 1966), the observation of cellular hypersensitivity to ionizing radiation in culture, and radioresistant DNA synthesis provided the basis for tests developed in several laboratories to aid in the diagnosis of A-T (Houldsworth and Lavin, 1980; Lavin and Schroeder, 1988; Painter and Young, 1980). However, none of these assays has been successful in identifying family members at risk, with the occasional exception of their application to prenatal diagnosis. An assay for radioresistant DNA synthesis has been used for prenatal diagnosis in A-T (Jaspers et al., 1981).

After identification of the *ATM* gene, diagnosis of A-T has tremendously improved by means of measuring ATM protein levels (Butch et al., 2004; Chun et al., 2003), ATM kinase activity, and mutation analysis of *ATM* (Chun and Gatti, 2004). Mutation detection for carrier and prenatal diagnosis, particularly in the light of specific mutations in the family, has become feasible, although there are a large number of described mutations widely distributed in *ATM.* Alternatively, it is possible to carry out immunoblotting or immunoassay, which has a considerably shorter turnaround time than immunoblotting, to detect ATM protein (Butch et al., 2004). Since most mutations in *ATM* are truncating and some missense mutations also destabilize the protein, ATM protein is either undetectable or reduced in amount in most A-T patients. In cases where mutant ATM protein is detectable, ATM kinase activity can be measured. This is determined by immunoprecipitation of ATM and assay using a substrate such as p53 and ^{32}P-ATP (Banin et al., 1998; Canman et al., 1998).

Mutation analysis of *ATM* has enabled definitive diagnosis of patients with manifestations consistent with A-T (hence presumed to have "A-T") or in patients who are classified as having "A-T variant" based on presence of atypical symptoms (Chun and Gatti, 2004). Now, A-T can reliably be distinguished, at the gene level, from the Mre11 deficiency (A-T–like disease), the oculomotor apraxia 1 (AOA1) (aprataxin deficiency) (Date et al., 2001, Moreira et al., 2001), the oculomotor apraxia 2 (AOA2) (senataxin deficiency) (Moreira et al., 2004), and other "A-T variants," including NBS (nibrin/Nbs1 deficiency) and $A\text{-}T_{Fresno}$. NBS manifests with microcephaly, mental retardation, apraxia, and telangiectasia in the absence of ataxia. On the other hand, $A\text{-}T_{Fresno}$ is a phenotype that combines the features of both NBS and A-T, with mutations in the *ATM* gene. Perhaps the closest to A-T is AOA2, which is characterized by progressive ataxia, choreoathetosis, and oculomotor apraxia. However, these patients do not have the immunodeficiency seen in A-T, nor the other laboratory markers; most importantly, they have normal *ATM* gene. Now that we are able to define the molecular defect, protein expression, and function in A-T, all patients with a "classical A-T" or "A-T variant" phenotype deserve genetic analysis to assign an accurate diagnosis, which would be instrumental in prognosis and genetic counseling.

A-T and Newborn Screening: Newborn screening using dried blood spots (DBS) for T cell receptor excision circles (TRECs) to identify severe combined immunodeficiencies (SCID) is increasingly being performed in the US (Mallot et al., 2013). It has recently been shown that more than half of A-T patients can actually be identified as abnormal by newborn screening for SCIDs because of the T lymphocytopenia which may be a feature of A-T in newborns. Subsequently, whole exome sequencing for mutation analysis performed in abnormal infants may enable an early genetic diagnosis of A-T. Early detection of the disease would be helpful in prevention of complications in the patient, and to

Table 47.5 LABORATORY TESTS TO SUPPORT DIAGNOSIS OF A-T

ASSAY	A-T PHENOTYPE	REFERENCE
Antibodies		
Serum IgA, IgE	Reduced	Thieffry et al. (1961)
Serum IgM	Monomeric form	Peterson et al. (1963)
Serum Proteins		
α-fetoprotein	Elevated	Waldmann and McIntire (1972)
Carcinoembryonic antigen	Elevated	Sugimoto et al. (1978)
Radiosensitivity		
Cell survival	Reduced	Taylor et al. (1976)
Chromosome aberrations	Elevated	Hecht et al. (1965); Higurashi and Conon (1973)
G2 phase delay	Exaggerated	Ford et al. (1984); Beamish and Lavin (1994)
p53 induction	Reduced/delayed	Kastan et al. (1992); Khanna and Lavin (1993)
Radioresistant DNA synthesis	Present	Houldsworth and Lavin (1980)

provide genetic counseling and carrier detection for the family (Mallot et al., 2013)

MUTATION-TARGETED THERAPY

In A-T, as in other genetic diseases, the mutations can be classified into four groups as nonsense, splicing, frameshift, and missense mutations. Each group of mutations can potentially be targeted with chemicals or oligonucleotides to correct the gene defect (Hu and Gatti, 2008).

NONSENSE MUTATIONS

Of 434 reported gene defects in A-T, 17 percent are nonsense *ATM* mutations, which create premature stop codons (UAA, UAG, or UGA) to terminate translation at that point, resulting in a truncated unstable protein (Hu and Gatti, 2008). Suppressing (i.e., reading through) the resulting premature stop codons with chemicals to allow translation to continue to the true end of the transcript is a promising approach to correct nonsense mutations. Proof of concept has been shown for aminoglycosides in a number of genetic diseases (Zingman et al., 2007). Aminoglycosides bind to the decoding site of the 16S ribosomal RNA. This binding results in a local conformational change that allows translation through what would otherwise be read as a premature termination codon. Paromomycin and G418 were the first aminoglycosides shown to partially restore the full-length protein of nonsense mutation in mammalian cells (Burke and Mogg, 1985). Gentamicin was tested in clinical trials for cystic fibrosis (Wilschanski et al., 2003), Duchenne muscular dystrophy (Politano et al., 2003), and hemophilia (James et al., 2005). Lai et al. (2004) studied aminoglycosides ex vivo in A-T lymphoblastoid cell lines. They used radiosensitivity, radioresistant DNA synthesis, and irradiation-induced autophosphorylation of ATM Ser-1981 to show that the aminoglycoside-induced full-length ATM protein was functional and corrected to various extents. Studies have shown some limitations of this approach: (1) true stop codons are not affected by the read-through compounds, (2) there is a need for high concentration of aminoglycosides to read through, which may be toxic to cells, and (3) most antibiotics do not cross the blood–brain barrier efficiently, which would compromise the use in central nervous system diseases. Paromomycin is a newly designed compound that seems to have an effective read-through activity at nontoxic concentrations (Pokrovskaya et al., 2010). PTC124 appears to be another small molecule, nonsense-suppression read-through compound shown to be effective in mouse models of Duchenne muscular dystrophy and cystic fibrosis (Du et al., 2008; Welch et al., 2007). To discover additional read-through chemicals, as well as structure–activity relationship (SAR) groups, Gatti's laboratory developed a high-throughput screen assay based on the protein truncation test (PTT) (Du et al., 2008). They have identified more than 50 nonaminoglycoside chemicals with read-through activity, some of which are promising for clinical use (Du et al., 2009).

SPLICE DEFECTS

Splicing mutations account for approximately 26 percent of the *ATM* gene defects, which affect pre-mRNA splicing. Antisense oligonucleotides have been successfully used to modulate splicing site selection (Vacek et al., 2003). Antisense oligonucleotides block the targeted splice sites and redirect the splicing machinery to a more appropriate nearby splice site by targeting pre-mRNA in a sequence-specific manner. Du et al. (2007) have used antisense morpholino oligonucleotides (AMOs) to redirect and restore normal splicing in the ATM gene for splicing mutations. High concentrations of antisense oligonucleotides are needed in the nucleus for efficiency of this approach. Recent improvements in the delivery vehicles, such as arginine-rich, cell-penetrating peptides (CPP) and nine-arginine tags fused to rabies virus glycoprotein peptides (RVG-9R), have rendered direct delivery of antisense oligonucleotides into mammalian leukocytes

and neurons (Kumar et al., 2007; Marshall et al., 2007). Du and Gatti (2011) have achieved considerable correction of intranuclear ATM protein levels with CPP-mediated AMOs. The fact that RVG-9R–mediated oligonucleotide delivery can cross the blood–brain barrier is also promising in A-T as well as in other genetic disorders with central nervous system involvement.

FRAMESHIFT MUTATIONS

Forty percent of *ATM* mutations are frameshifts resulting from nucleotide insertions or deletions (Hu and Gatti, 2008). Gene therapy, antisense oligonucleotide–mediated exon skipping, and revertant mosaicism approaches are potentially promising to correct a frameshift mutation in A-T.

MISSENSE MUTATIONS

Missense and in-frame mutations that produce proteins with variable functions represent about 16 percent of *ATM* mutations (Hu and Gatti, 2008). Different therapeutic approaches can be developed based on the resultant function of the protein with small molecule compounds and chimeraplast, which are RNA/DNA oligonucleotides.

Mutation-targeted approaches in A-T, as well as in other primary immunodeficiency disorders, are definitely promising in treatment.

A-T HETEROZYGOTES AND ELEVATED CANCER RISK

Although A-T heterozygotes do not exhibit symptoms of the disease, they do have radiosensitivity to a lesser extent than homozygotes, a finding first reported by Chen et al. (1978). A number of other assays have been used to confirm this finding (Chen et al., 1994; Paterson et al., 1985; Roisin and Ochs, 1986; Rudolph et al., 1989; Shiloh et al., 1986). However, there is considerable variation among heterozygotes, and none of the assays is sufficiently reliable for A-T carrier diagnosis without reference to a known homozygote relative.

A Hardy-Weinberg estimate for heterozygotes using the early data of Boder and Sedgwick (1970) predicts a frequency of 1 in 100 individuals, whereas that of Swift et al. (1986) would give a value between 0.3 and 0.4 per 100. Because A-T is autosomal recessive, it would be expected that heterozygotes would show no symptoms. However, mortality from cancer among blood relatives of A-T patients is higher than expected. Swift et al. (1991) found an increased cancer risk in 1,599 adult blood relatives of A-T patients from 161 A-T families, compared to 821 of their spouses as controls. A subgroup of 294 obligate heterozygotes had relative risks of 3.8-fold above controls for men and 3.5-fold for women for all types of cancer. The relative risk for breast cancer in women was 5.1-fold. Several other epidemiological studies also support an increased risk of breast cancer in A-T heterozygotes but with smaller relative risk (Geoffroy-Perez et al., 2001; Inskip et al., 1999; Janin et al., 1999; Olsen et al., 2001; Pippard et al., 1988). Molecular genotyping and mutation analysis have produced apparently conflicting data on the incidence of *ATM* mutations in different cohorts of breast-cancer patients. In A-T family studies, Athma et al. (1996) detected 25 heterozygotes among 33 women with breast cancer, where only 15 were expected by chance alone. Fitzgerald et al. (1997) detected heterozygous mutations in 2 of 202 healthy women with no history of cancer, whereas the figure was 2 of 410 for women with early-onset breast cancer. Other smaller studies support these observations (Bay et al., 1998; Bebb et al., 1999; Chen et al., 1998). However, Broeks et al. (2000) detected 7 germline *ATM* mutations in 82 breast-cancer patients with either early-onset disease or bilateral breast cancer. In most of these studies truncating mutations were screened for, since they represent the bulk of mutations seen in A-T patients. One explanation for the disparate findings might reside in possible differences between truncating and missense mutations in *ATM*. Indeed, Gatti et al. (1999) proposed a model to explain the relationship between ATM mutations and cancer susceptibility discriminating between missense and truncating mutations. They envisaged that an *ATM* missense mutant expressing mutant protein could act in a dominant-negative capacity to interfere with normal ATM function. There is evidence that missense mutations in the kinase domain of *ATM* have a dominant interfering effect (Lim et al., 2000). Recent evidence for an increased frequency of *ATM* missense mutations in breast-cancer patients adds support for this model (Broeks et al., 2000; Dörk et al., 2001; Izatt et al., 1999; Teraoka et al., 2001).

Swift et al. (1991) asked whether diagnostic or occupational exposure to ionizing radiation increases the risk of breast cancer in women heterozygous for A-T. However, Norman and Withers (1993), estimating that annual mammography for 35 years would give a woman a total exposure of only 10 cGy, argued that on the basis of an increased sensitivity to radiation on the order of 30 percent (Cole et al., 1988), the lifetime risk for breast cancer from mammography would be 2 cases per 100 women. This would not significantly add to the natural lifetime risk of 1 in 9 in the general population. Additional studies of cancer and heterozygous *ATM* mutation are needed that also address the potential combined effects of mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* as well as better-defined environmental risk factors.

FUTURE DIRECTIONS

Our understanding of A-T has expanded enormously since the defective gene *ATM* was cloned. However, the more we learn, the more we become aware that we know little. With the advent of large-scale proteomic analyses, the number of potential substrates for the ATM protein has risen up to 700, meaning that there is still much more to learn about A-T and the ATM. Clearly, ATM is involved in many different signaling pathways, and it may have a regulatory role in crucial cellular functions not only in the nucleus but also in the cytoplasm. Challenges ahead include defining the substrates, establishing

the physiological importance of ATM-mediated phosphorylations, and assembling these events into signaling pathways. Better understanding of these mechanisms will be helpful in elucidating the connections between the lack of ATM and the various abnormalities in A-T to develop novel treatment strategies. More importantly, the role of ATM in more common diseases with high impact on public health, such as diabetes and cancer, will be better defined, paving the way to target ATM for global therapeutic purposes.

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48.

CHROMOSOMAL INSTABILITY SYNDROMES OTHER THAN ATAXIA-TELANGIECTASIA

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A number of syndromes exist in which an increased incidence of spontaneous and/or induced chromosomal aberrations is the unifying diagnostic criterion. These disorders, called *chromosomal instability syndromes,* include the classical chromosomal instability syndromes—namely, ataxia-telangiectasia (A-T), Fanconi anemia (FA), and Bloom syndrome (BS). On the basis of this definition, Nijmegen breakage syndrome (NBS) and A-T–like disorder (A-TLD) must also be added to this group. More recently, case reports of patients with deficiencies of ligase I (LIG1), ligase IV (LIG4), nonhomologous end-joining (NHEJ), and RAD50 have provided evidence for the existence of a long list of diseases belonging to this group.

There is growing evidence that the following genes mutated in chromosomal instability syndromes are involved in protecting—directly or indirectly—human genome integrity by contributing to the complex regulation of double-strand break (DSB) repair: *ATM* in A-T, *NBS1* in NBS, *BLM* in BS, *MRE11* in A-TLD, *Lig1* in ligase I deficiency, *Lig4* in ligase IV deficiencies, *NHEJ1* in Cernunnos deficiency, *RAD50*, and the group of at least 13 genes known to be affected in the heterogeneous disorder of FA. Recent studies of protein–protein interactions suggest that the normal gene products associated with most of these genetic defects are in fact involved in forming or regulating a huge protein complex active in the surveillance and maintenance of genomic integrity. Results from these studies indicate that the gene products of *ATM*, *BLM*, *NBS1*, *MRE11*, and *RAD50* are all members of a complex named *BASC* (*BRCA1*-associated genome surveillance complex). They might appear as a single protein subunit or as a stable subcomplex such as nibrin, MRE11, and RAD50 (Wang et al., 2000). As described below, most of the known FA genes may be also linked indirectly or even directly to the BASC complex (Ahmad et al., 2002).

Most of these syndromes exhibit features of immunodeficiency or at least hematological symptoms resulting from a clinically relevant deficiency of cells belonging to the immune system. In this chapter, the term *immunodeficiency* is used in this broader sense. The triad of immunodeficiency, neoplasia, and infertility is typical for most of the syndromes associated with chromosomal instability. This observation can best be explained by defects in the complex system of DSB repair, affecting either NHEJ or homologous rejoining (HR). Thus tumor development in these syndromes is a direct consequence of defective DNA repair, which leads to mutations of cancer susceptibility genes acting as gatekeepers or caretakers (Duker, 2002; Kinzler and Vogelstein, 1997). Since meiotic recombination requires the production and repair of DSBs, the high incidence of infertility observed in patients with chromosomal instability syndromes is not surprising. Because the production of immunoglobulins relies on the network of proteins supporting the process of NHEJ (reviewed in van Gent et al., 2001), failure of a member of this group of proteins could lead to immunodeficiency. Thus, although primary immunodeficiency is not a typical symptom of chromosomal instability syndromes, these disorders may further our understanding of the relationship between DSB induction, DSB repair, and immunoglobulin production.

In this chapter, we will focus on three clinically defined syndromes—FA, NBS, and BS—followed by a short presentation of more recently discovered chromosomal instability syndromes that show A-T–like or NBS-like phenotypes due to specific gene mutations (*Mre11*, *Rad50*, *Lig1*, *Lig4*, *NHEJ1*). Other diseases, for example Rothmund-Thomson syndrome (MIM 268400), Werner syndrome (MIM 277700) (Table 48.1), and Riddle syndrome (MIM 611943), will either be briefly described or are beyond the scope of this text because the clinical presentation does not suggest a defect of the immune system or published data are insufficient to classify them as

Table 48.1 FEATURES OF CHROMOSOMAL INSTABILITY SYNDROMES

	A-T	A-TLD	NBS	NHEJ1	LIG4	RAD50	LIG1	BLOOM	WRNS	FA	RS-SCID
					General Data						
OMIM No.	208900	604391	251260	611291	606593	604040	126391	210900	277700	227650	605988
Gene	*ATM*	*MRE11*	*NBS1*	NHEJ1	*LIG4*	*RAD50*	*LIG1*	*BLM*	*WRN*	>11 genes	*DCLRE1C*
Gene product	ATM	Mre11	Nibrin	DNA repair factor Cernunnos	Ligase IV	Rad50	Ligase I	RECQL2-helicase	RECQL3-helicase		Artemis
Gene Locus	11q23	11q21	8q21	2q35	13q22–q34	5q31	19q13.2–13.3	15q26.1	8p12		10p
					Clinical Manifestations						
Growth retardation	+	+	+	+	+	+	+	+	+	+	+
Microcephaly	–	–	+	+	+	+		+		+	
Facial anomalies	–	–	+	+	+	+		+			
Receding forehead			+	+		+					
Receding mandible			+	+		+		+			
Disturbances of hair growth			+								
Scleral telangiectasia Cataract	+	–	(+)		–	–	+		+		
Skin abnormalities	+	–	+		+	+	+	+	+		
Photosensitivity	+	–	+		+		+	+			
Cutaneous telangiectasias	+	–	+		+		+	+	+		
Pigmentation defects			+			+		+		+	
Neurological abnormalities	+	+	–		–	+					
Cerebellar ataxia	+	+	–			–					
Oculomotor apraxia	+	+	–			–					
Choreathetosis	+	+	–			–					
Mental retardation	–	–	(+)	(+)	+	–				(–)	
Other manifestations	+	–	+	+	+	–					
Infertility	+		+	?	+		+	+		+	
Skeletal anomalies			+	+	+					+	
Renal anomalies			+	+						+	
Infections	+	–	+	+	+	–	+				+
Malignancies	+	–	+		+	?	+	+	+	+	+
Lymphoma			+				+	+	+		+
Leukemia			+		+			+	+	+	
Osteosarcoma	+								+		
					Laboratory Manifestations						
Immunodeficiency	+	–	+	+	+	–	+	+			+
Pancytopenia				+	+			+		+	
Humoral immunodeficiency	+		+	+						–	
Cellular immunodeficiency	(+)		+	+						–	
Elevated AFP	+	–	–	–		–				–	
Cytogenetic Abnormalities	+	+	+		+	+	+	+	+	+	+

(continued)

Table 48.1 (CONTINUED)

	A-T	A-TLD	NBS	NHEJ1	LIG4	RAD50	LIG1	BLOOM	WRNS	FA	RS-SCID
Spontaneous chromosomal instability	+	+	+		?	+		+		+	
Chromosome 7 and 14 rearrangements	+	+	+			(+)		−		−	
Increased translocation frequencies	+		+	+		+		+	+	+	
Hypersensitivity to ionizing radiation/ bleomycin	+	+	+	+	+	+	+		−	−	+
Hypersensitivity to UV light							+				
Hypersensitivity to alkylating agents	+		+			+	+		−	+	
Radioresistant DNA synthesis	+		+	−		+				−	
Increased SCE frequency	−	−	−	−	(+)	−		+		−	

AFP, α-fetoprotein; A-T, ataxia-telangiectasia; A-TLD, ataxia-telangiectasia–like disease; Bloom, Bloom syndrome; FA, Fanconi anemia; LIG1, ligase I deficiency; LIG4, ligase IV deficiency; NBS, Nijmegen breakage syndrome; NHEJ1, non-homologous end-joining factor 1 (NHEJ1) responsible for SCID with microcephaly and chromosomal instability; RAD50, RAD50-deficiency; RS-SCID, radiosensitive severe combined immunodeficiency syndrome; UV, ultraviolet; WRNS, Werner Syndrome.

immunodeficiency diseases. Chapter 47 has been dedicated to A-T, a chromosomal instability syndrome with specific expression of immunodeficiency. Moreover, subgroups of patients with severe combined immunodeficiency (SCID) expressing radiosensitivity, and most probably also chromosomal instability, are excluded from this chapter. This subgroup of SCID is presented in greater detail in Chapter 13.

FANCONI ANEMIA

FA (MIM 227650) is an autosomal recessive chromosome instability syndrome characterized by bone marrow failure and an increased risk of neoplasia, particularly leukemia. In addition, a range of congenital abnormalities, such as growth retardation, radius aplasia, and hyperpigmentation of the skin, are observed in some patients.

The clinical presentation of FA is highly variable. As is often the case, the more severe phenotypes have been overemphasized, while one third of FA patients have no congenital anomalies (Giampietro et al., 1997; Glanz and Fraser, 1982). The generally accepted critical diagnostic criterion for FA is an increase in chromosome breakage after in vitro treatment of patient cells, usually peripheral blood lymphocytes, with bifunctional alkylating agents such as mitomycin C (MMC) (Sasaki and Tonomura, 1973).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Hematological Complications

The bone marrow of FA patients deteriorates, usually within the first decade of life, although this aspect varies considerably. Thrombocytopenia and macrocytosis, for example increased erythrocyte volume (mean cell volume > 100 fl) associated with increased fetal hemoglobin levels, are followed by granulocytopenia, leukopenia, and eventually pancytopenia. Although aplastic anemia is accompanied by recurrent infections, FA is clearly a genetic hematological disorder rather than a primary immunodeficiency. Pancytopenia, however, results in a clinically relevant deficiency of cells considered to be part of the immune system.

Acute myeloblastic leukemia occurs 15, 000 times more frequently in FA patients than in the general population. In addition, squamous cell carcinoma of the skin and gastrointestinal tract are frequent complications. The true cancer risk is difficult to estimate, since some patients die prematurely from infections or as a consequence of bone marrow transplantation; one estimate suggests that at least 15 percent of FA patients develop neoplasia (Alter, 1996).

CYTOGENETICS

The involvement of the FA gene(s) in the "metabolism and mechanics of the chromosome" was postulated by Schroeder and German (1974) on the basis of characteristic chromosome breaks observed in lymphocytes of FA patients (Fig. 48.1). These breaks are particularly chromatid breaks, and translocations involve nonhomologous chromosomes, leading to triradial and quadriradial forms. This increased somatic mutation rate in lymphocytes of FA patients is thought to explain the predisposition to acute myelogenic leukemia and other malignancies. A highly specific feature is a hypersensitivity to cross-linking agents. This phenomenon is still used as the diagnostic marker for FA when a gene mutation cannot be proven. Detailed protocols describing common cytogenetic techniques are provided by Wegner and Stumm (1999).

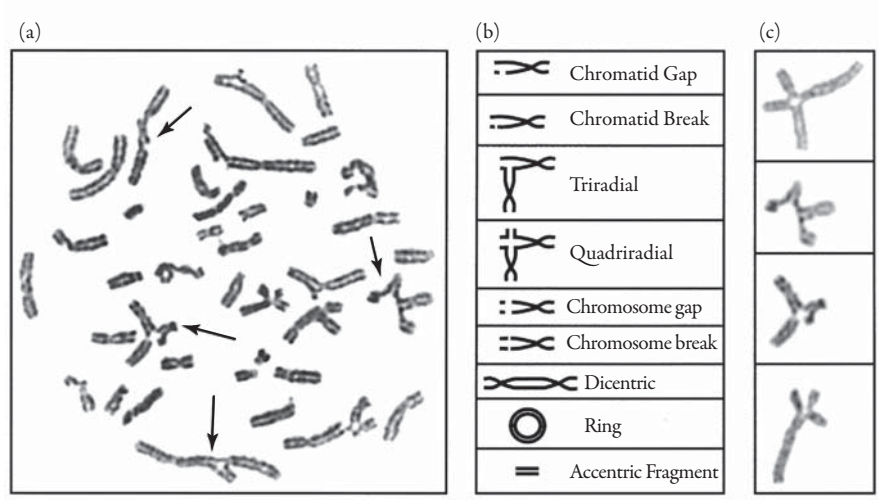

Figure 48.1 Chromosomal instability in Fanconi anemia (FA). (A) Lymphocyte chromosomes of a patient with Fanconi anemia. Only some of the chromatid breaks in this massively damaged metaphase are indicated. (B) Schematic representation of the chromosome aberrations seen in lymphocyte chromosomes from patients with chromosomal instability syndromes. (C) Examples of characteristic chromosome reunion figures seen in FA patients.

The observation that peripheral blood lymphocytes from a subgroup of FA patients apparently contain two populations of cells was first reported in 1983 by Kwee et al. Increasing doses of MMC shift one population to higher levels of chromosome damage, while a second population remains as unaffected as wild-type cells. Although chromosome breakage analysis probably does not detect low-level mosaicism due to reversion of the genomic mutation, such cases can be revealed by the isolation of MMC-resistant Epstein-Barr virus (EBV)-immortalized lymphoblasts. On the basis of this criterion, up to 25 percent of all FA patients are likely to be mosaic (Lo Ten Foe et al., 1997). In all mosaic patients analyzed, skin fibroblasts show the characteristic chromosome instability, suggesting that the reversion events leading to a wild-type cell population are specific for lymphocyte progenitors (Gregory et al., 2001;Waisfisz et al., 1999a). The high incidence of mosaics may require cytogenetic analysis of fibroblasts from patients suspected of having FA but no increased breakage in standard lymphocyte cultures.

CELL CYCLE

Analysis of the cell cycle in FA has consistently shown increased numbers of cells in G2 phase (Seyschab et al., 1993), where they are presumably arrested to repair DNA lesions. Treating FA cells with cross-linkers increases the proportion of cells in G2 and has become an alternative and/or substantiating diagnostic criterion for FA. This disturbance of the cell cycle explains the poor growth of FA cells in vitro.

Interestingly, the slow growth of FA cells and their accumulation in G2 can be normalized by keeping the cell cultures at reduced oxygen tension (Schindler and Hoehn, 1988). Even the spontaneous chromosomal breakage is lost at 5 percent O_2 (Joenje et al., 1981). These and other findings have led to the suggestion that the primary defect in FA is related to the avoidance of oxygen radicals or to the repair of the DNA lesions caused by these highly reactive molecules.

GENETICS

FA is genetically heterogeneous, and this heterogeneity can be assessed by somatic cell fusion and analysis of cross-linker sensitivity in the cell hybrids. Currently 13 complementation groups are known; these are denoted by the abbreviation *FA* followed by a letter—for example, FA-A, FA-B. FA-A is the largest group (approximately 60 percent), followed by FA-G (approximately 10 percent) and FA-C (approximately 10 percent). Identification of the underlying gene has been achieved for all of the groups, mostly by the technique of functional cloning by selecting cDNAs that enable patient cells to survive an otherwise lethal dose of MMC. The genes are denoted by the abbreviation *FANC* followed by a letter—for example, *FANCA, FANCG.* In some cases a chromosomal localization for the gene had been previously established by linkage analysis in families (Pronk et al., 1995; Saar et al., 1998; Waisfisz et al., 1999b). After the demonstration by Garcia-Higuera et al. (2001) that FANCD2 interacts with BRCA1, *BRCA2* was identified as an FA gene after analysis of FA-B and FA-D1 patients for mutations in this gene (Howlett et al., 2002). *FANCL* (Meetei et al., 2003), *FANCM* (Meetei et al., 2005), and the X-chromosomal gene *FANCB* (Meetei et al., 2004) were identified through the finding that many FA proteins interact to form a stable complex. *FANCJ* was identified by a candidate gene approach as the BRCA1 binding protein BRIP1 (Levran et al., 2005). The current status of gene identification is given in Table 48.2, together with some details of the known FA genes.

While many FA genes were novel when they were identified, some were previously known or showed significant homology to known genes. *FANCG* proved to be identical to the gene *XRCC9* (de Winter et al., 1998), which had been

Table 48.2 **FA COMPLEMENTATION GROUPS AND GENES**

FA-GROUP	PREVALENCE (%)	FANCD2 MONO-UBIQUITINYLATION?	GENE	CHROMOSOME	EXONS	PROTEIN SIZE (kDa)
FA-A	60	no	*FANCA*	16q24.3	43	163
FA-B	rare	no	*FANCB*	Xp22.31	10	95
FA-C	15	no	*FANCC*	9q22.3	14	65
FA-D1	5	yes	*FANCD1/BRCA2*	13q12–13	27	384
FA-D2	5	no	*FANCD2*	3p25.3	44	155
FA-E	rare	no	*FANCE*	6p21–22	10	59
FA-F	rare	no	*FANCF*	11p15	1	42
FA-G	10	no	*FANCG/XRCC9*	9p13	14	68
FA-I	rare	yes	*FANCI*	15q25–26	37	150
FA-J	rare	yes	*FANCJ/BRIP1*	17q23.2	20	130
FA-L	rare	no	*FANCL*	2p16.1	14	52
FA-M	rare	no	*FANCM*	14q21.2	23	250
FA-N	rare	yes	*FACN/ PALB2*	16p12.1	13	131

isolated through its ability to correct (or cross-complement) an X-ray–sensitive hamster cell mutant from complementation group 9 (Liu et al., 1997a). *FANCD1* is identical to *BRCA2* and *FANCJ* is a BRCA1 binding protein. *FANCM* shows homology to the archaeal *Hef* gene that has ATP-dependent helicase activities and is involved in DNA repair (Meetei et al., 2005). *FANCD2* shows homology with sequences in lower organisms, such as *Drosophila* (Timmers et al., 2001). The protein products of the *FANCD2* and *FANCI* genes are rather unusual among FA proteins, as discussed below. Other functional motifs have so far not been identified in FA proteins.

Despite repeated efforts, a correlation between complementation group and clinical features or course of the disease has not generally been possible. Patients with and without congenital abnormalities are found in all groups with sufficient numbers of patients for analysis. Leukemia seems to occur earlier in FA-G patients than in FA-A or FA-C patients (Faivre et al., 2000).

MUTATION ANALYSIS

The FA genes have been extensively analyzed for mutations. Most patients are compound heterozygotes with private mutations, but there are also founder mutations in the three largest groups, FA-A, FA-C, and FA-G. Whereas particular deletions of *FANCA* exons are common in the Afrikaner population in South Africa (Tipping et al., 2001), a nonsense mutation, E105X, accounts for 44 percent of mutant *FANCG* alleles in Germany (Demuth et al., 2000). In *FANCC,* the mutation IVS4 + 4a > t is homozygous in the majority of Ashkenazi Jewish FA patients and is associated with a particularly severe disease in terms of both congenital abnormalities and hematopoietic failure (Whitney et al., 1993). In contrast, Japanese patients with the same mutation are not severely affected (Futaki et al., 2000). This finding indicates the strong influence of genetic background on disease manifestation and explains the difficulties in establishing a genotype–phenotype correlation, even within one complementation group. On the whole, truncation mutations strongly outweigh missense mutations in the FA genes. *FANCA* in particular has many deletions due to the Alu-repeat elements in and around the gene (Levran et al., 1998). So far, no null mutations have been observed in FANCD2, and cells from all patients show residual protein that is monoubiquitinated after DNA damage. Nevertheless, FANCD2 mutation correlates with a more severe clinical course in comparison to the other groups (Kalb et al., 2007).

The biallelic mutations in the *BRCA2/FANCD1* gene found in FA-D1 patients are hypomorphic (Howlett et al., 2002). Presumably the amorphic mutations are embryonically lethal in the homozygous state, as in *Brca2* null-mutant mice (Ludwig et al., 1997).

For diagnostic purposes, the complementation group can be established by fusion of patient cells to reference cell lines or by retroviral transfer of the known FA genes, followed by examination of cross-linker sensitivity. DNA from patients assigned to a complementation group can be analyzed for mutations, and this information is then available for further diagnoses in the family, including prenatal diagnosis. The ongoing collection of clinical data and mutation analyses in Europe and the United States is facilitating attempts at a genotype–phenotype correlation (Faivre et al., 2000; Gillio et al., 1997).

FA MOSAICS

The molecular basis of reversion has been elucidated for at least six mosaic cases (Lo Ten Foe et al., 1997; Waisfisz et al., 1999a). In one case, mitotic recombination with a breakpoint within the *FANCC* gene placed both mutations on one allele and generated a heterozygous cell. In another patient, one mutation was lost in the reverted cells; retention of heterozygosity indicated that gene conversion was probably responsible for this reversion. The same mechanism was responsible

for reversion in cells of this patient's affected brother. In three additional cases, all homozygous for *FANCA* or *FANCC* mutations, reversion was due to additional de novo mutations in *cis*. Thus, a single base deletion was corrected by two further single base deletions that restored the reading frame. Similarly, a 5 bp insertion corrected the effects of an inherited single base insertion. Finally, a C>T transition corrected a missense mutation. This latter correction was found independently in two affected mosaic siblings, suggesting a specific molecular mechanism, whereas the other reversions are presumably due to random insertion or deletion events and selection.

Although reversions have been shown to occur in lymphohematopoietic stem cells and may be associated with a seemingly milder hematological disease, bone marrow failure and leukemia have been reported for several patients with revertant mosaicism (Gregory et al., 2001).

MOLECULAR BIOLOGY OF FA

The chromosome breakage observed in FA is specific for agents like MMC, which are capable of intercalating into a DNA double helix and forming covalent cross-links between the two strands. Thus the DNA lesion to which FA cells are particularly sensitive is the interstrand cross-link. The hypothesis based on this observation postulates that FA cells are deficient in the repair of DNA cross-links.

The similarity in the cellular and clinical phenotype within the complementation groups was taken as evidence for involvement of the FA proteins in a common pathway. Indeed, association of the FA proteins in multimeric complexes has been proven by immunoprecipitation studies and by yeast two-hybrid analyses (Medhurst et al., 2001). In the nucleus, most FA proteins join to form the FA core complex (Fig. 48.2) (de Winter et al., 2000; Meetei et al., 2003, 2004).

A direct link to DNA repair processes in the cell was not made until the cloning of *FANCG*, which was found to be identical to the gene mutated in UV40, a hamster cell DNA-repair mutant, and implicated in postreplicational repair (Busch et al., 1996; Liu et al., 1997a). Further and even stronger evidence for a role in DNA repair was provided by the cloning of the *FANCD2* gene and the discovery that its protein is monoubiquitinated as a response to DNA damage and relocates to discrete nuclear foci, where it associates with BRCA1 (Garcia-Higuera et al., 2001). These nuclear foci are almost certainly sites where DNA lesions (e.g., DSBs, cross-links) are being actively repaired (see also the section on NBS, below). A further FA protein, FANCI, is also monoubiquitinated by the FA core complex and colocalizes to FANCD2 nuclear foci (Smogorzewska et al., 2007). While monoubiquitination of FACNI requires monoubiquitination of FANCD2, the modification of FANCD2 is independent of FANCI monoubiquitination. FANCI is phosphorylated by ATM and/or ATR.

BRCA1 is a binding partner of BRCA2/FANCD1, and both of these are required for the recruitment of RAD51, the RecA homolog, to the sites of DSBs for error-free repair by homologous recombination and gene conversion. BRCA2/FANCD1-deficient cells have been shown to be inefficient in the formation of RAD51 foci (Yuan et al., 1999).

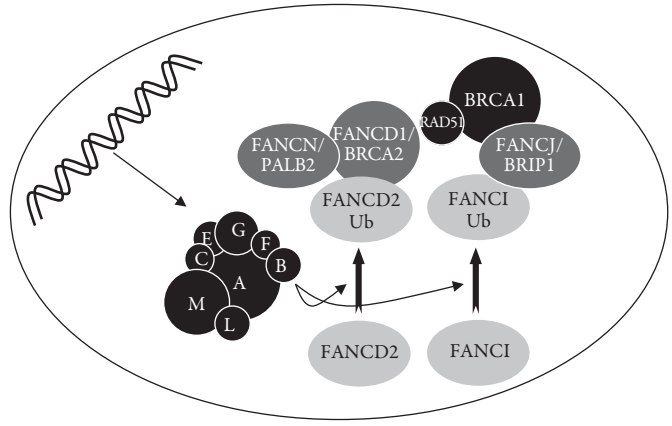

Figure 48.2 Network of FA proteins. A highly simplified schematic representation of interactions of the FA gene products. A core complex of eight of the FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) is required for monoubiquitination of further FA proteins, FANCD2 and FANCI, in response to DNA damage. Modified FANCD2 and FANCI then relocate to the sites of DNA damage, where they associate with BRCA1 and BRCA2, which is identical to FANCD1. Other proteins, FANCN and FACNJ, are not involved in the modification of FANCD2 and FANCI. BRCA1 and BRCA2 bind the important effector of homologous recombination, RAD51.

This finding correlates with a loss of repair by gene conversion, an increase in repair by alternative error-prone pathways, and extreme cross-linker–induced chromosomal instability (Tutt et al., 2001). The finding that the FA protein complex of FANCA, FANCB, FANCC, FANCG, FANCE, FANCF, and FANCL is required for modification of the FANCD2 protein (Fig. 48.2) has linked these proteins nicely into the same pathway (Garcia-Higuera et al., 2001; Meetei et al., 2003, 2004; Pace et al., 2002). The finding that RAD51 foci formation is reduced in FA cells from all complementation groups suggests that monoubiquitinated FANCD2, like BRCA1 and BRCA2/FANCD1, is involved in RAD51-mediated DNA repair (Digweed et al., 2002b). Furthermore, FANCD2 has been shown to interact directly with BRCA2/FANCD1 (Hussain et al., 2004). Figure 48.2 shows a model for these protein interactions.

The reliance on error-prone DNA repair pathways in the other FA complementation groups may lead to the characteristic chromosomal instability of FA and be a direct result of the inability to monoubiquitinate FANCD2 and FANCDI and efficiently recruit RAD51 for repair by gene conversion. Convincing data have been presented showing reduced homologous recombination in chicken DT40 cells mutated in *FANCG* (Yamamoto et al., 2003) and *FANCC* (Niedzwiedz et al., 2004). Similarly, using sophisticated plasmid reporters, defects in homologous recombination have been demonstrated in human and mouse cells with mutations in *FANCA*, *FANCD2*, and *FANCG* (Nakanishi et al., 2005; Yang et al., 2005). In contrast to previous studies, in mutant mouse and human cells the error-prone repair pathway of single-strand annealing (SSA) was also affected, suggesting further roles for the monoubiquitinated FANCD2 protein. It is possible, even likely, that the individual FA proteins are involved in

further interactions and that these may influence their activity in monoubiquitination of FANCD2. Thus, the demonstrated oxygen sensitivity and cell-cycle disturbance described in FA cells have yet to be incorporated logically into the FA pathway or, perhaps more appropriately, into the FA network. Interestingly, a functional link between FANCD2 and the NBS1/MRE11/RAD50 pathway has been indicated by several reports (Digweed et al., 2002a; Nakanishi et al., 2002; Pichierri et al., 2002). Upon replicative stress, FANCD2 localizes to discrete spots on mitotic chromosomes, indicating that the FA pathway is involved in the repair or rescue of damaged cells in anaphase and telophase of mitosis (Naim and Rosselli, 2009).

ANIMAL MODELS

Knockout mice for the *Fanca*, *Fancc*, and *Fancg* genes have been produced but show few of the disease symptoms observed in patients (Cheng et al., 2000; Whitney et al., 1996; Yang et al., 2001). They do not have skeletal malformations, nor, surprisingly, do they show anemia or increased neoplasia. Although cells from these animals show the characteristic chromosomal breakage and cross-linker sensitivity, the animals are hematologically healthy unless treated with cross-linkers, in which case they suffer bone marrow failure. The only feature that is similar to the human clinical phenotype is reduced fertility. Perhaps not surprisingly, double-knockout *Fanca/Fancc* mice are not more severely affected than the single-knockout animals, indicating that these genes probably do not have further functions over and above those of the protein core complex (Noll et al., 2002).

Spontaneous defective hematopoiesis has been reported in double-knockout mice, with disruptions in both *Fancc* and the Cu/Zn superoxide dismutase genes (Hadjur et al., 2001). These results provide evidence that abnormal regulation of the cellular redox state in FA may be involved in the bone marrow failure of patients. Increased tumorigenesis was reported for a *Fancc/p53* double-knockout mouse with a spectrum of malignancies similar to those of FA patients (Freie et al., 2003). Increased frequencies of epithelial tumors were reported for the *Fancd2* knockout mouse, suggesting perhaps a phenotypic overlap with hypomorphic *Brca2/Fancd1* mice (Houghtaling et al., 2003).

TREATMENT AND PROGNOSIS

Several treatment possibilities are available for patients with FA. Erythrocyte and thrombocyte transfusions can compensate for bone marrow failure, although hemosiderosis is a concern. The bone marrow abnormalities of many patients respond to androgen and cytokine therapy, even if only temporarily. Allogeneic bone marrow transplantation from a histocompatible sibling can cure the bone marrow failure. Transplantations from matched unrelated donors have had poor outcomes in FA (MacMillan et al., 2000). Bone marrow transplantation is complicated by the increased sensitivity of FA patients to pretransplant conditioning treatment. However, new cytoreductive regimens, particularly those including fludarabine, and the use of T-cell depletion, are encouraging (Kapelushnik et al., 1997; MacMillan and Wagner, 2010; McCloy et al., 2001; Stepensky et al., 2011; Thakar et al., 2011) and have improved outcome regardless of donor source. The occurrence of expanding clonal aberrations in bone marrow, particularly those involving chromosome 3q, has been shown to correlate with a poor prognosis (Tönnies et al., 2003).

The relative accessibility of hematopoietic stem cells made FA an obvious candidate disorder for gene therapy (reviewed by Tolar et all., 2012), and the first trials were conducted in 1997 (Liu et al., 1997b, 1999). The expectation was that corrected stem cells would have a strong selective advantage in the hypoplastic bone marrow of FA patients, as suggested by the finding of mosaic patients in whom total hematopoiesis is driven by a reverted clone. Gene transfer experiments with the knockout *Fancc* mouse have shown that positive selection can indeed be achieved (Gush et al., 2000). Currently the main obstacle to the development of gene therapy in FA is the poor efficiency of gene transfer into hematopoietic stem cells. A promising new approach to gene therapy in FA may be the correction of hematopoietic stem cells generated from patient-derived induced pluripotent stem cells (Raya et al., 2009).

FUTURE DIRECTIONS

The cloning of most of the FA genes and the final demonstration of a link to DNA repair have changed the course of current FA research. It is realistic to expect that much more detailed information on this novel network for DNA repair will be forthcoming. The availability of mouse models will enable the evaluation of treatment proposals including gene therapy, when gene transfer methodology is advanced enough to make this an alternative therapy for FA patients. In the meantime, the considerable improvement in bone marrow transplantation from unrelated donors must be extrapolated to the treatment of FA patients, and new therapies for the treatment of squamous cell carcinoma in FA patients have to be developed. The finding that modification of the FANCD2 protein does not occur in cells from most FA patients after DNA damage may be exploited in the future as a biochemical assay for diagnosis of the disease. Finally, the role of FA genes in the occurrence of malignancy in the general population will have to be addressed, in view of reports that indicate a role for *FANCF* silencing in acute myeloid leukemia (Tischkowitz et al., 2003) and cervical cancer (Narayan et al., 2004).

NIJMEGEN BREAKAGE SYNDROME

NBS (MIM 251260), a rare autosomal recessive disorder belonging to the group of chromosomal instability syndromes, was first described by a group of investigators at the University of Nijmegen (Hustinx et al., 1979) and has been recently reviewed in great detail (Chrzanowska et al., 2012). The clinical hallmarks of NBS patients are severe microcephaly, growth retardation, typical facial appearance, combined immunodeficiency, radiosensitivity, and an increased cancer risk. On the cellular level, the NBS abnormalities are closely related

to those observed in A-T patients. Cells of both syndromes reveal chromosomal instability, a marked sensitivity to ionizing irradiation (IR) and radiomimetic agents, a radioresistant DNA synthesis (RDS), and additional defects in cellular checkpoint control. On the molecular genetic level, however, both syndromes are clearly distinguishable from each other. Whereas A-T is caused by mutations in the *ATM* gene located on chromosome 11q23 (Savitsky et al., 1995), NBS is caused by mutations in the *NBS1* gene localized on chromosome 8q21 (Varon et al., 1998). The *NBS1* gene product, nibrin, is part of the hRAD50/hMRE11/nibrin complex (Carney et al., 1998), which seems to function in concert with ATM in a damage-response pathway that affects a DNA repair process as well as cell-cycle checkpoint control (Girard et al., 2000).

DEMOGRAPHICS

Whereas in the first edition of this text the number of diagnosed NBS patients amounted to 58, this figure increased to approximately 170 patients at the end of 2004. Detailed clinical data on 55 patients collected in the NBS Registry in Nijmegen are included in the report of an international study group (International Nijmegen Breakage Syndrome Study Group, 2000). While single NBS patients were reported from North America (Bakhshi et al., 2003; Cerosaletti et al., 1998), Chile (Pincheira et al., 1998), Bosnia (Kleier et al., 2000), Argentina (Rosenzweig et al., 2001), Morocco (Maraschio et al., 2001), Turkey (Tekin et al., 2002), Yugoslavia (Pasic, 2002), and Italy (Barth et al., 2003), the majority of NBS patients known thus far are of Slavic origin (particularly of Polish and Czech descent, with 83 and 35 patients identified to date, respectively, and of Russian descent [Resnick et al., 2002]) and carry a common founder mutation 657del5 in exon 6 of the *NBS1* gene (Varon et al., 1998, 2000). The prevalence of this founder mutation was estimated by Varon et al. (2000) in the Czech Republic, Poland, and the Ukraine by the screening of Guthrie cards. A mean prevalence of 1:177 for NBS heterozygotes was found in these three populations. The highest prevalence was found in the Czech population (1:154), followed by the Ukraine (1:182) and Poland (1:198). However, marked regional differences were observed in Poland, ranging from 1:90 to 1:314. Further studies performed recently on populations of three large regions in Poland established a mean prevalence of 1:166 for the 657del5 mutation in Poland (Cybulski et al., 2004; Steffen et al., 2004; M. Mosor, personal communication). All of these frequencies were much higher than the prevalence of 1:866 reported for the same mutation in Germany (Carlomagno et al., 1999). On the basis of these data, the prevalence for NBS homozygotes in the Czech Republic can be estimated at 1 per 95,000. Interestingly, the actual frequency of 1 per 271,000 is much lower than expected. The most likely explanation for this discrepancy is the underdiagnosis of NBS patients because of the rarity of NBS and its relatively mild phenotype. This assumption is corroborated by two observations: (1) the late diagnosis of many NBS patients, often in cases where a malignancy requires examinations that finally disclose the presence of NBS, and (2) detection of 3 new NBS patients in a cohort of 23 Czech patients with primary microcephaly tested for the major Czech mutation, the 657del5, in the *NBS1* gene (Seeman et al., 2004). This underdiagnosis is likely a general problem worldwide; thus, more attention should be spent on the correct diagnosis of this disease.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Since the first reports on two siblings from Nijmegen (Hustinx et al., 1979; Weemaes et al., 1981), NBS has been recognized as a multisystem disorder similar to A-T. The main clinical manifestations (Table 48.3) indicate that various tissues and organs are affected, including brain, skin, blood, and gonads.

Growth and Development

Microcephaly, the most striking symptom of the disorder, has been observed in the great majority of children at birth (van der Burgt et al., 1996). Those who are born with a normal head circumference (OFC) will develop progressive and severe microcephaly during the first months of life. The primary cause of small head size in NBS patients is the genetically determined stunted growth of the brain (see Pathological Findings, below). Thus, the observed premature closing of the sutures and fontanels can be considered a developmental consequence (no increased intracranial pressure was noted, except in the two cases with coexisting hydrocephalus). Among the observed Polish patients, the decrease in OFC ranged from −9.0 SDs to −4.4 SDs, whereas the proportions among the diminished head measurements (i.e., length and breadth) were retained. Only one case with a normal OFC has been described (Chrzanowska et al., 2001). Growth retardation may occur prenatally in some NBS patients, but in most cases birth weight and length correlate to gestational age. After an initial period of distinct growth retardation lasting from birth to about the second year of life, a slight improvement of growth rate (body height and weight, but not head circumference) is usually observed. Most affected individuals show growth below or around the third percentile, whereas a few

Table 48.3 **CLINICAL FEATURES OF NIJMEGEN BREAKAGE SYNDROME**

Microcephaly—severe and progressive
Characteristic face
Sloping forehead and receding mandible
Prominent midface
Retardation of statural growth
Ovarian failure
Immunodeficiency
Predisposition to malignancies
Intelligence level
Normal or borderline in early childhood
Progressive deficiency of IQ scores in later life

patients achieve a height around the 10th or even 25th percentile (Chrzanowska et al., 2001; International Nijmegen Breakage Syndrome Study Group, 2000). Intestinal malabsorption, cardiac defects, or hormonal abnormalities related to thyroid or pituitary gland functions have all been excluded as a cause of the growth retardation.

Sexual maturation. Longitudinal studies of growth and development of Polish NBS patients, of whom three females and six males reached puberty, drew attention to the lack of development of secondary sexual characteristics in teenage girls. They presented with primary amenorrhea, absent breast development, and scanty pubic or axillary hair. In two of the three girls, all older than 18 years, repeated pelvic ultrasonography revealed small ovaries, resembling streak gonads, and infantile uteri. All three had markedly elevated plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and very low estradiol levels, indicating primary ovarian failure (Chrzanowska et al., 1996). A study of the pituitary-gonadal axis performed on a large group of Polish patients, including 25 females, confirmed the preliminary findings predicting a very high incidence of ovarian failure in this syndrome. Markedly elevated plasma concentrations of FSH, in relation to age, and very low estradiol levels (hypergonadotropic hypogonadism) were found in all but one of the tested females. Serum FSH concentrations exceeding 30 IU/L, a value indicative of ovarian failure, were documented in the majority (76 percent of those tested), both at pubertal and prepubertal ages (Chrzanowska, 1999; Chrzanowska et al., 2000). Single cases of hypergonadotropic hypogonadism had been reported earlier, including a 21-year-old woman with NBS phenotype (Conley at al., 1986) and a now 30-year-old woman described earlier (Wegner et al., 1988) who subsequently developed primary amenorrhea. In males there was only a slight delay in onset of puberty, and the levels of gonadotropins and testosterone corresponded to Tanner's classification of sexual maturity stages (Chrzanowska, 1999). Offspring from affected subjects have never been reported, and firm conclusions on fertility are hampered by the young age of the patients and their short lifespan. Thus, hypergonadotropic hypogonadism in females, indicative of either ovarian dysgenesis or hypoplasia, must be included in the clinical spectrum of NBS.

Psychomotor development and behavior. In general, developmental milestones are reached at expected times during the first years of life. Patients with normal intelligence (Barbi et al., 1991; Green et al., 1995; Seemanová et al., 1985; Wegner et al., 1988) or mental retardation of variable degree (Chrzanowska et al., 1995a; Conley et al., 1986; Stoppa-Lyonnet et al., 1992; Weemaes et al., 1981) have been reported. Follow-up studies of Polish patients showed that the level of intellectual function decreases with age. Most of the children tested in infancy and early childhood had IQ scores indicating a normal or borderline intelligence with striking psychomotor hyperactivity. When tested or retested after the age of 7, difficulties in concentration became more pronounced. At school age, lower levels of intellectual function were observed and became more evident in subjects over 14 years of age, when all tested patients were mildly or moderately delayed (Chrzanowska, 1999). Most of the mentally retarded patients need educational support and should attend special education classes or schools. All of the children have a gentle and cheerful personality and, despite being shy, are usually capable of good social interactions.

Craniofacial manifestations. The craniofacial characteristics become more obvious with age because of the severe and progressive microcephaly. The facial appearance is very similar among NBS patients and is characterized by a sloping forehead and receding mandible, prominent midface with a relatively long nose (slightly beaked in most patients or upturned in some), and upward slanting of palpebral fissures (Fig. 48.3). In some individuals the ears seem to be relatively large and dysplastic.

Other Manifestations

Central nervous system malformations. Developmental abnormalities of the brain are relatively frequent and appear to be more common than expected. Partial agenesis of the corpus callosum was documented in one third (6/18) of Polish patients who underwent cranial magnetic resonance imaging (MRI) (Bekiesinska-Figatowska et al., 2000, 2004; Chrzanowska et al., 2001) as well as in at least two others (Maraschio et al., 2001; Resnick et al., 2002). Agenesis of the corpus callosum was associated developmentally with colpocephaly (i.e., disproportionate enlargement of the trigones, occipital horns, and usually temporal horns of the lateral ventricles). Large collections of cerebrospinal fluid (arachnoid cysts) in the parieto-occipital and/or occipitotemporal regions were found in three patients (Bekiesinska-Figatowska et al., 2000; Chrzanowska et al., 2001; Stoppa-Lyonnet et al., 1992). The cysts resulted from an anomalous splitting of the arachnoid membrane and are a congenital anomaly of the developing subarachnoid system. Both defects, callosal hypoplasia and arachnoid cysts, may frequently be underdiagnosed in NBS patients because they are asymptomatic. Hydrocephalus was reported in several patients, including one pair of siblings (Bekiesinska-Figatowska et al., 2000; Muschke et al., 2004; Taalman et al., 1989). Neuronal migration disorder in the form of schizencephaly and focal pachygyria has been diagnosed each in a single patient (Bekiesiriska-Figatowska et al., 2000; Der Kaloustian et al., 1996).

Skin and vascular anomalies. Skin pigmentation abnormalities expressed as café-au-lait–like spots (rather irregular in shape) and/or depigmented spots can be seen in most patients; in three Polish patients, progressive vitiligo was observed at adolescence. Sun sensitivity of the eyelids is less frequent. Multiple pigmented nevi and cavernous or flat hemangiomas occur in a proportion of patients (Chrzanowska, 1999; International Nijmegen Breakage Syndrome Study Group, 2000; Peréz-Vera et al., 1997).

Skeletal anomalies. Minor skeletal defects such as clinodactyly of the fifth fingers and/or partial syndactyly of the second and third toes are encountered in about half of the patients (International Nijmegen Breakage Syndrome Study Group, 2000); less common is hip dysplasia (~15 percent) (Chrzanowska, 1999, and unpublished observation). Uni- or bilateral preaxial polydactyly was observed in four patients (Chrzanowska et al., 1995a; Maraschio et al., 2001), and

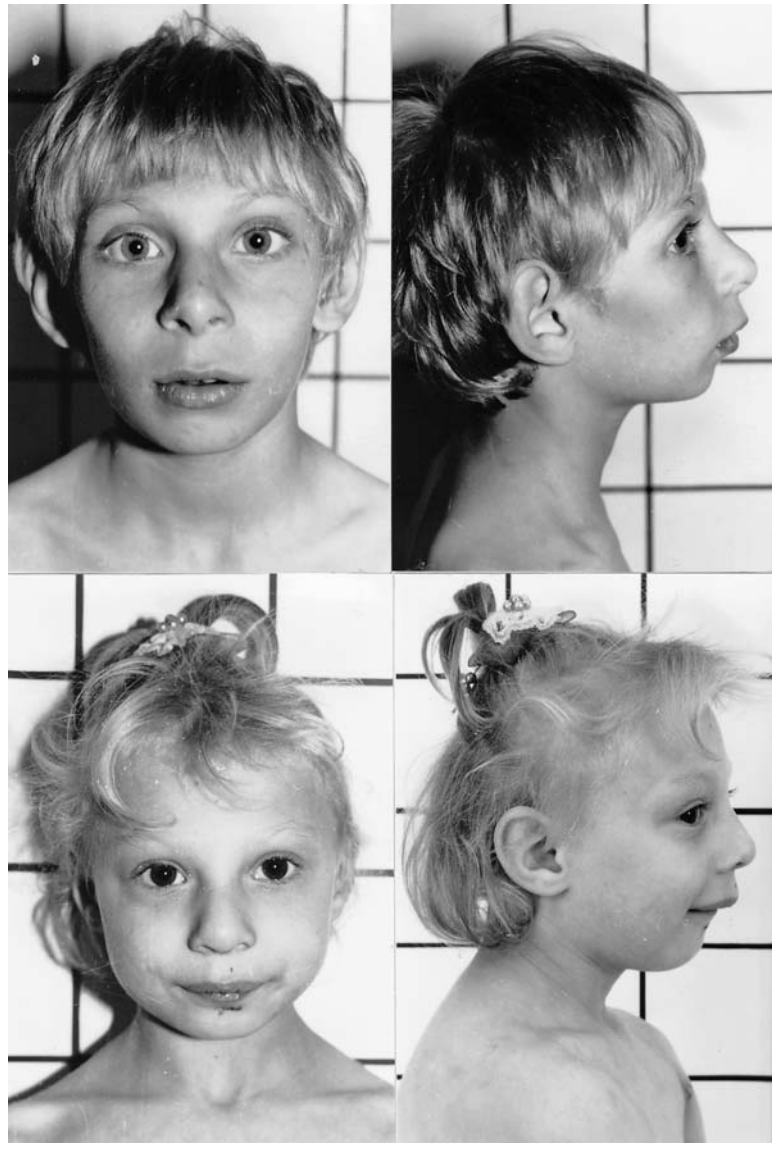

Figure 48.3 Characteristic facial features of a 7-year-old male patient with Nijmegen breakage syndrome (upper row) and his 5-year-old sister. Note microcephaly, microgenia, sloping forehead, slightly upward slanting of palpebral fissures, and prominent midface.

sacral agenesis was found in one (K. Chrzanowska, unpublished observation).

Urogenital, anal, and miscellaneous malformations. Anal atresia or stenosis was noted in several cases (Chrzanowska, 1999; Tekin et al., 2002; Wegner et al., 1988). Among the urogenital anomalies, ectopic single kidney or dystopic kidneys were diagnosed in eight patients (Chrzanowska, 1999; International Nijmegen Breakage Syndrome Study Group, 2000; Muschke et al., 2004) and hydronephrosis in three additional patients (Seemanová et al., 1985; Taalman et al., 1989). Congenital cardiovascular or heart defects were noted in two cases (Chrzanowska et al., 2001; Tekin et al., 2002). Hypospadias (Der Kaloustian et al., 1996), cryptorchism (Pincheira et al., 1998), and genitourinary fistula (Chrzanowska, 1999) were observed each in only a single patient, as were other miscellaneous congenital anomalies such as cleft lip/palate (Seemanová et al., 1985), choanal atresia (Seemanová et al., 1985), and tracheal hypoplasia (Chrzanowska, 1999). Ultrasonographic evaluation of a large group of Polish patients revealed a high frequency of polysplenia (20 percent), a peculiarity with no clinical significance (Chrzanowska, 1999).

Infections and Autoimmune Disorders

As in A-T (see Chapter 47), the extent of immunodeficiency or proneness to infection in NBS patients showed striking intra- and interfamilial variability. Respiratory tract infections were present in most children. Recurrent pneumonia and bronchitis may result in bronchiectasis, respiratory insufficiency, and premature death from respiratory failure (Seemanová et al., 1985; Weemaes et al., 1981). Meningitis, sinusitis, and otitis media with draining ears and mastoiditis were observed in some children, as were gastrointestinal infections with diarrhea and urinary tract infections. Opportunistic infections are very rare, as in A-T (International Nijmegen Breakage

Syndrome Study Group, 2000). Other diseases, probably caused by a defective immune system, have been observed in single cases: autoimmune hemolytic anemia, hemolytic anemia followed by thrombocytopenia, childhood sarcoidosis with ocular and cutaneous manifestations (Chrzanowska, 1999), and a juvenile rheumatoid arthritis-like polyarthritis (Rosenzweig et al., 2001). In two unrelated Polish patients, coexistence of NBS and Gilbert syndrome (familial idiopathic unconjugated hyperbilirubinemia associated with a partial reduction of hepatic glucuronyl transferase activity) has been noted (Chrzanowska, 1999).

Predisposition to Malignancies

Both the immunodeficiency and the chromosome instability may predispose NBS patients to tumor development at an early age. In A-T, approximately 10 to 15 percent of the homozygotes develop malignancy in early adulthood (Taylor et al., 1996). The rate is even higher in NBS. Cancer before the age of 21 years was noted in 40 percent (22/55) of the patients included in the NBS Registry in Nijmegen (International Nijmegen Breakage Syndrome Study Group, 2000). Updates from the two largest national NBS registries, the Polish registry (K. Chrzanowska, unpublished data) and the Czech registry (Seemanová et al., reported at the International Workshop on NBS, Prague, Czech Republic, 2002), indicated cancer development in 54 percent (45/83) and 65 percent (19/29) of patients, respectively. The great majority of malignancies were of lymphoid origin, found in 41 and 15 patients, respectively. In the Polish series, the most frequent malignancies were non-Hodgkin's lymphomas (NHL) in 30 patients (those of B-cell origin slightly exceeded those of T-cell origin), followed by lymphoblastic leukemia/lymphoma in 8 patients (T-LBL/ALL, T-ALL, pre-B-ALL) and Hodgkin's disease in 3 patients (Gladkowska-Dura et al., 2000). Acute myeloblastic leukemia (AML) has been diagnosed in two NBS patients (Resnick et al., 2002; K. Chrzanowska, unpublished observation). In a recent report from the Polish NBS Registry, 56 of 105 patients enrolled as of October 2007 had developed primary malignancies (53 percent), including 51 cases of lymphoma. Over half of those were classified as diffuse large B-cell lymphoma (DLBCL), followed by lymphoblastic T-cell lymphoma (T-LBL) and rarely Burkitt's and classic Hodgkin's lymphoma (Gladkowska-Dura et al., 2008).The first case of T-cell prolymphocytic leukemia (T-PLL) in NBS was reported by Michallet et al. in 2003.

Several patients are known to have developed a second malignancy. One German patient (Rischewski et al., 2000) and four Polish patients had two consecutive lymphomas of the same type, with complete remission of 5 to 7 years in between (Gladkowska-Dura et al., 2000, 2008). Two additional Polish patients developed a different type of lymphoma as a second event: in one case, the initial Burkitt-like lymphoma was followed by a DLBCL, and in the other case, a peripheral T-cell lymphoma developed 3 years after the DLBCL (Gladkowska-Dura et al., 2008). Two other patients (Dutch and Czech) developed a different type of tumor after 10 and 4 years of remission, respectively (Weemaes et al., 2002). One Polish patient was recently diagnosed to have a third consecutive lymphoma of the same type (time interval between the two relapses, 7 and 6 years, respectively; Gladkowska-Dura et al., 2005). Both concordance of tumor type in a pair of NBS siblings (Wegner, 1991) and discordance in another pair (Wegner et al., 1999) have been reported.

Solid tumors have been less frequently noted, probably because the tumors usually develop at an older age. Five patients have developed a medulloblastoma (Chrzanowska et al., 1997; Bakhshi et al., 2003; Distel et al., 2003; E. Seemanová, personal communication; F. Tzortzatou-Stathopoulou, personal communication) and two a rhabdomyosarcoma (Der Kaloustian et al., 1996; Tekin et al., 2002). Other tumors are represented only in single cases and include malignant meningioma, gonadoblastoma, Ewing sarcoma (E. Seemanova, personal communication), and ganglioneuroblastoma (K. Chrzanowska, unpublished observation).

At least three patients who had medulloblastoma and received radiation therapy before being diagnosed with NBS were fatally injured and eventually died from complications of the therapy (Bakhshi et al., 2003; Chrzanowska et al., 1997; Distel et al., 2003). Alopecia was a side effect observed in a Polish patient with AML given an 18-Gy dose of cranial irradiation for central nervous system (CNS) prophylaxis. However, another Polish patient with T-cell acute lymphoblastic leukemia (T-ALL) tolerated well an identical dose of prophylactic cranial irradiation. Both patients were treated for malignancy before the diagnosis of NBS was established (K. Chrzanowska, unpublished observation).

Most malignancies develop before the age of 20 years (median age, 9 years; range, 1–34 years), and in about 20 to 30 percent of patients cancer appears prior to the diagnosis of NBS (Varon et al., 2000). On the basis of available records, an approximately 50-fold risk of early onset of cancer and a more than 1,000-fold risk of lymphoma are estimated for patients with NBS.

The precise mechanisms leading to the increased cancer prevalence in NBS remain unclear. Thus it is an open question as to whether the *NBS1* gene has a general function as a tumor-suppressor gene. Molecular investigation of the frequency of the most common *NBS1* mutation, 657del5, in two NHL cohorts with 109 and 62 German children, respectively, failed to detect this mutation in the *NBS1* gene (Rischewski et al., 2000; Stanulla et al., 2000). Furthermore, a fluorescence in situ hybridization (FISH) analysis of tumor samples from 16 German NHL patients detected no deletions of the *NBS1* gene (Stumm et al., 2001b). Sequencing studies on 20 Japanese lymphomas (Hama et al., 2000) and tumor samples from 91 NHL patients in the United States (Cerosaletti et al., 2002) provided no evidence that *NBS1* mutations play a major role in the development of NHL. These studies demonstrate that neither a NBS heterozygote status nor mutations or deletions of the *NBS1* gene in the tumor itself are frequent events in patients with B- and T-cell lymphomas. However, a Polish study of 456 children with lymphoid malignancies (208 with NHL and 248 with ALL) revealed five heterozygous carriers of a germline 657del5 mutation, in contrast to the expected incidence of 2.75 (Chrzanowska et al., 2004). This study has

been expanded, showing similar results and suggesting that *NBS1* gene heterozygosity is not a major factor in lymphoid malignancies in childhood and adolescence (Chrzanowska et al., 2006).

In contrast to NHL, Varon et al. (2001) detected mutations in *NBS1* in 15 percent of cases of childhood ALL, whereas none was observed in controls, a finding indicating a possible involvement of the *NBS1* gene in the development of ALL. There is evidence that heterozygotes for NBS also show a significantly increased rate of malignancies (Seemanová et al., 1990). Recently performed studies on 1,683 nonselected Polish patients with malignant tumors detected increased germline mutation frequencies for the 657del5 mutation and increased frequencies for the R215W amino acid exchange variant, indicating that heterozygous carriers of NBS mutations may indeed have an increased risk of developing tumors, especially in breast cancer and melanoma (Steffen et al., 2004). These data have been corroborated by increased translocation frequencies in NBS heterozygotes (Stumm et al., 2001a; see Cytogenetics, below). More recently Shimada et al. (2004) reported the first case of aplastic anemia with a homozygous missense mutation in the *NBS1* gene (I171V) and hypothesized that the *NBS1* gene may play an important role in the pathogenesis of this complication. A major analysis of cancer occurrence in NBS families has recently unequivocally demonstrated an increased cancer risk for heterozygotes with the Slavic mutation (Seemanova et al., 2007).

PATHOLOGICAL FINDINGS

Of more than 70 NBS patients whose deaths have been ascertained, less than 10 died from infections that led to fatal respiratory failure, and two from renal insufficiency due to amyloidosis. Two others died as a result of bone marrow aplasia (Resnick et al., 2002), a hallmark of another chromosomal instability syndrome, FA. All remaining patients died from malignancies. The oldest survivors with the Slavic 657del 5 mutation were a 33-year-old Polish patient and a 31-year-old Dutch patient, both men. A patient with a homozygous dinucleotide insertion in exon 7 was alive at 55 years of age without having developed a malignancy (Maraschio et al., 1986; Varon et al., 2006). The mutation in this patient leads to an alternatively spliced mRNA and a truncated protein product with an internal deletion that, presumably, upholds the functions of the full-length protein (Varon et al., 2006).

Autopsies performed on several NBS patients showed a brain weight that was more than 50 percent reduced in all patients examined and internal hydrocephalus in some (Muschke et al., 2004; Seemanová et al., 1985; van de Kaa et al., 1994). A clear neuropathological difference from A-T has been demonstrated in NBS with a cerebellum of normal size and development (van de Kaa et al., 1994).

A simplified gyral pattern, especially in the frontal lobes, with a severely diminished number of neocortical neurons has been documented (Lammens et al., 2003). Marked hypoplasia or absence of the thymus, or thymus replacement by fibrous tissues was reported in several cases (Muschke et al., 2004; Seemanová et al., 1985; van de Kaa et al., 1994).

Suspicion of a lymphoproliferative disorder is the most frequent indication for lymph node biopsy, but only a few reports have appeared on the histological and immunophenotypic features of lymphomas (Elenitoba-Johnson and Jaffe, 1997; Paulli et al., 2000; van de Kaa et al., 1994). A detailed description of 10 cases of NHL and 1 of Hodgkin's disease revealed morphological and immunotypic diversity of these NBS-linked lymphomas, ranging from immature, precursor-type lymphoid malignancies to mature T-cell lymphomas to immunoglobulin-producing, diffuse large B-cell lymphomas (DLBCL), a spectrum that appears to be more characteristic for adult rather than pediatric patients (Gladkowska-Dura et al., 2000, 2002, 2008). Further immunophenotypic and rearrangement studies have revealed clonal *IgH* gene rearrangements in all but one DLBCL case, clonal *IgK* gene rearrangements in all cases, and *IgL* gene rearrangements in two (Gladkowska-Dura et al., 2002, 2008). None of the cases showed a Bcl-2/IgH translocation.

LABORATORY FINDINGS

Laboratory tests helpful in the diagnosis of NBS include evaluation of humoral and cellular immunity, quantitation of serum α-fetoprotein (AFP) levels, karyotyping, and radiosensitivity assays. Molecular analysis is possible in most cases (see Genetics, below).

Immunological Data

The immune deficiency in NBS patients is very heterogenic and affects the humoral and cellular immune systems (Table 48.4). A longitudinal follow-up study of 40 NBS patients, diagnosed and monitored at a single medical center, the Children's Memorial Health Institute (CMHI) in Warsaw, offered a unique opportunity to gather clinical and laboratory data on a large series of patients (Gregorek et al., 2002). This study extends and supplements data collected earlier to assess the immune system in individuals with NBS (Chrzanowska et al., 1995b; International Nijmegen Breakage Syndrome Study Group, 2000).

Table 48.4 **IMMUNE DEFECTS IN PATIENTS WITH ATAXIA-TELANGIECTASIA VARIANT OR NIJMEGEN BREAKAGE SYNDROME**

Lymphopenia, T and B cells
Humoral immunity
Absent or low serum levels of IgA, IgG, IgM, or IgE
Abnormal IgG subclass distribution (deficient in IgG2, IgG4)
Cellular immunity
Low numbers of CD4+ subset
Decreased CD4+/CD8+ ratio
High number of NK cells
Impaired proliferative response to mitogens in vitro

The most important observation is the considerable variability in immunodeficiency seen among different patients and in the same individual over the course of time (Gregorek et al., 2002). At diagnosis, severe hypogammaglobulinemia with IgG concentrations below 2.0 g/L, low or undetectable IgA, and decreased IgM was observed in 21 percent of patients, while an additional 20 percent had normal concentrations of all three major immunoglobulin classes. The most common finding was a combined deficit of IgG and IgA (31 percent), followed by isolated IgG deficiency (28 percent). IgG subclass distribution was abnormal in most patients, affecting predominantly IgG4 (74 percent), followed by IgG2 (66 percent) and IgG1 (63 percent). In about 37 percent of NBS patients, normal levels of total serum IgG can mask deficiency of IgG subclasses. This phenomenon points to the importance of determining IgG subclasses, especially in NBS patients who suffer from frequent infections. The concentrations of IgM and IgG3 are rarely affected; in fact, IgM levels may be elevated (Chrzanowska et al., 1995b; Gregorek et al., 2002; Wegner et al., 1988). These observations suggest that the process of class switching to C_H genes downstream of γ3 (γ1, α1, γ2, γ4, ε, and α2) is frequently blocked in NBS. Recently, Pan et al. (2002) provided the first evidence linking ATM and nibrin to class-switch recombination (CSR). Lahdesmaki et al. (2004) have suggested that MRE11, nibrin, and ATM might play both common and independent roles in CSR.

Similar to A-T patients, both normal (Der Kaloustian et al., 1996; Green et al., 1995) and disturbed (Weemaes et al., 1991; Wegner et al., 1988) antibody responses to diphtheria and tetanus vaccination have been reported. One NBS patient failed to mount a primary antibody response to *Helix pomatia* hemocyanin (Weemaes et al., 1991). Naturally acquired IgG-specific antibodies to most invasive pneumococcal polysaccharides (e.g., serotypes 3, 19, 23) were not detectable or were found in very low titers in 75 percent of patients investigated at CMHI. Moreover, only 25 percent of patients vaccinated against hepatitis B virus developed antibodies to hepatitis B surface antigen of the protective IgG isotype, which are restricted to two IgG subclasses: IgG1 (present in all positive samples) and IgG3 (found in one patient only). In 65 percent of the individuals, only IgM antibodies to hepatitis B surface antigens were found; in the remaining 10 percent, no specific antibodies were detectable, despite normal or only moderately decreased levels of total IgG (Gregorek et al., 2002). Systematic longitudinal observations of 17 available NBS patients at CMHI showed a progressive deterioration of the immune system in 9 of 17 patients (53 percent). There was no correlation between immunodeficiency profile (or its severity) and duration of the disease or the age or gender of the patients studied.

T-cell immunity is abnormal in most tested patients. The most consistent defects are reduced numbers of $CD3^+$ T cells observed in 93 percent, a reduced number of $CD4^+$ T cells in 95 percent, and a reduction in $CD8^+$ T cells in 80 percent. A decreased CD4/CD8 ratio is also a characteristic feature of this syndrome (Chrzanowska et al., 1995b; International Nijmegen Breakage Syndrome Study Group, 2000; van der Burgt et al., 1996). The number of B cells is reduced in most patients (≈75 percent) but may also be normal or even elevated in some patients despite marked deficiency of serum immunoglobulins, suggesting an intrinsic B-cell defect affecting CSR (Gregorek et al., 2002). Detailed characteristics of natural killer (NK) and T-lymphocyte surface receptors have been reported in a large group (n = 42) of NBS patients (Michalkiewicz et al., 2003).

A deficiency of $CD4^+$, $CD45RA^+$ (naïve) cells and an excess of $CD4^+$, $CD45RO^+$ (memory) T cells has been observed in all patients tested (Chrzanowska et al., 1995b). A relatively high number of NK cells was noted in the majority of NBS patients (Der Kaloustian et al., 1996; Green et al., 1995). In vitro lymphocyte proliferation in response to mitogens is defective in most patients (Conley et al., 1986; Chrzanowska et al., 1995b; Der Kaloustian et al., 1986; International Nijmegen Breakage Syndrome Study Group, 2000; Seemanová et al., 1985; Weemaes et al., 1981; Wegner et al., 1988). In one case lymphocyte proliferation in the presence of specific antigens (tetanus, tuberculin, candidin) was absent despite a normal response to PHA (Stoppa-Lyonnet et al., 1992). Recall antigens (Merieux) given intradermally produced no delayed-type hypersensitivity reactions (Wegner et al., 1988).

α-Fetoprotein Level

Serum AFP concentration is within the normal range in NBS (van der Burgt et al., 1996), in contrast to A-T, where elevated levels are found in more than 90 percent of patients (Woods and Taylor, 1992).

Cytogenetics

In most cases, the diagnosis of NBS is entertained on clinical findings and confirmed by cytogenetic and/or molecular analysis of the *NBS1* gene. T lymphocytes of NBS patients often show a poor response to mitogens; consequently, cultures exhibit a low mitotic index. This limitation to cytogenetic characterization of patient's cells, particularly when testing for mutagenic sensitivity, can be overcome in most cases by using EBV-transformed lymphoblastoid cell lines.

NBS cells express the typical cytogenetic features of a chromosomal instability syndrome (Table 48.5). In general, NBS patients have a normal karyotype, but chromosomal instability, as in A-T patients, is a consistent finding. One of the most striking features of NBS is the high level of chromosome rearrangements in cultured T lymphocytes involving chromosomes 7 and 14. Most of these rearrangements occur in chromosome bands 7p13, 7q35, 14q11, and 14q32 (as in A-T), which are the location of the human immunoglobulin and T-cell–receptor genes (Aurias et al., 1980; Aurias and Dutrillaux, 1986). Generally, translocations are detectable in 10 to 35 percent of NBS cells and in 5 to 10 percent of A-T cells (van der Burgt et al., 1996). The most frequently detected aberration in T lymphocytes is inv(7)(p13q35), followed by other rearrangements such as t(7; 14)(p13; q11), t(7; 14)(q35; q11), t(7; 7)(p13; q35), and t(14; 14)(q11; q32) (van der Burgt et al., 1996). Other less frequently reported NBS

Table 48.5 CYTOGENETIC AND CELLULAR FEATURES OF NIJMEGEN BREAKAGE SYNDROME CELLS

Normal constitutive karyotype
Increased spontaneous chromosomal instability
Open chromatid and chromosome breaks
Rearrangements of chromosomes 7 and 14
Telomere fusions
Radioresistant DNA synthesis
Hypersensitivity to ionizing radiation and radiomimetic agents
Increased sensitivity to alkylating agents

breakpoints, for example t(X;14)(q27–28; q11–13) , have also been found in A-T cells (Wegner, 1991).

Open chromosomal aberrations, such as chromatid breaks, chromosome breaks, and centric fragments, as well as marker chromosomes and unspecific chromatid exchanges have been frequently found in lymphocytes and fibroblasts of NBS patients (Barbi et al., 1991; Chrzanowska et al., 1995a, 2001; Conley et al., 1986; Demas et al., 1981; Der Kaloustian et al., 1996; Kleier et al., 2000; Maraschio et al., 2001; Pérez-Vera et al, 1997; Seemanová et al., 1985; Stoppa-Lyonnet et al., 1992; Taalmann et al., 1989; Tupler et al., 1997; Wegner et al., 1988). In contrast, chromosomal instability has not been observed in NBS bone marrow cells (Weemaes et al., 1981). An additional uncommon chromosomal abnormality has been described in a 5-year-old NBS patient who showed, in addition to the typical 7/14 translocations, monosomies of nearly all chromosomes in 64 percent of the analyzed lymphocyte metaphases (Der Kaloustian et al., 1996). This finding points to deficiencies in the fidelity of mitotic chromosome separation. Whether nibrin, the product of *NBS1,* is involved in chromosome segregations remains unclear. An elevated rate of spontaneous chromosomal instability has been observed in EBV-positive immortalized B lymphoblasts from many NBS patients (Conley et al., 1986; Maraschio et al., 2001; Tupler et al., 1997).

However, some NBS cell lines have a low frequency of chromosomal aberrations or no chromosomal instability at all (Chrzanowska et al., 2001; Stumm et al., 1997; Wegner, 1991). In contrast to primary T lymphocytes, the spontaneous instability in lymphoblastoid cell lines (LCLs) is expressed as unspecific chromosomal aberrations, including a tendency of chromosomes to form telomeric associations resulting in dicentric chromosomes. Molecular and cellular data collected from NBS patients suggest that this cytogenetic phenomenon is a direct consequence of a functional deficiency of nibrin, a protein found at telomeres, where it associates with the telomeric repeat binding factor TRF2 (Lombard and Guarente, 2000; Zhu et al., 2000). Chromosomes from NBS patients have shortened telomeres that can be corrected by reintroduction of nibrin into patient fibroblasts (Ranganathan et al., 2001). Thus, the absence of nibrin might interfere with telomere metabolism, resulting in illegitimate fusion of telomeres between different chromosomes. Siwicki et al. (2003) have provided evidence that telomere length maintenance is intact in T lymphocytes in the absence of full-length nibrin, presumably because of an alternatively spliced NBS protein of 70 kDa.

All of the cytogenetic aberrations discussed above were obtained by standard microscopic analysis of Giemsa-stained and/or GTG-banded chromosomes. More distinct insight into the chromosomal instability of NBS cells was obtained by use of FISH with a three-color whole-chromosome painting assay (WCP 1, 2, 4) (Color Plate 48.I). Through use of this technique, an increased frequency of spontaneous translocations was detected (Stumm et al., 2001a). The presence of chromosomal aberrations, which might escape detection by conventional cytogenetic techniques, demonstrates that the degree of spontaneous genomic instability in NBS might be even higher than previously thought and may be an important risk factor for tumor development. The WCP 1, 2, 4 assay is also suitable for the detection of radiation-induced chromosomal instability in NBS and A-T patients (Neubauer et al., 2002).

Sister chromatid exchange (SCE) frequencies were found to be normal in lymphocytes (Barbi et al., 1991; Conley et al., 1986; Der Kaloustian et al., 1996; Weemaes et al., 1981; Wegner et al., 1988), fibroblasts, and LCLs (Conley et al., 1986) from all NBS patients investigated to date. The induction of chromosomal breakage in NBS lymphocytes and fibroblasts by irradiation proves a strong hypersensitivity to ionizing radiation (IR) as well as to radiomimetic agents such as bleomycin (Green et al., 1995; Taalman et al., 1989; Wegner et al., 1988). These characteristics clearly separate NBS cells from normal cells (Fig. 48.4) and have been used in our laboratories to confirm the diagnosis. A metaphase with a typical spectrum of lesions is shown in Figure 48.5. Increased radiosensitivity of NBS cells is also apparent from a decrease in colony-forming

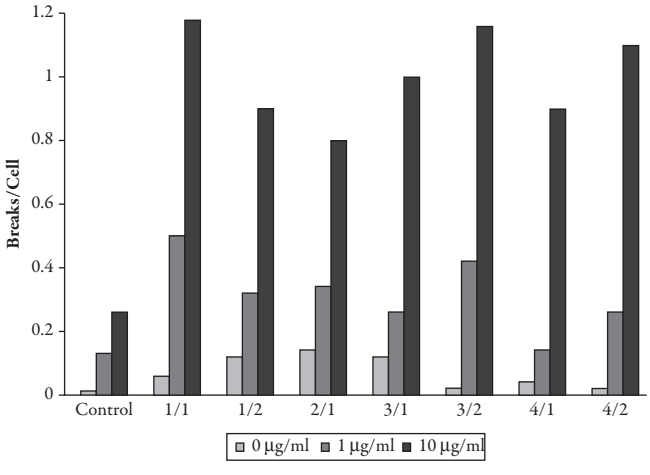

Figure 48.4 Spontaneous and bleomycin-induced chromosomal breakage rates in lymphoblastoid cell line metaphases from Nijmegen breakage syndrome (NBS) patients and a control, respectively. Bleomycin was added 1 hour before harvesting: 50 cells/column were counted in each situation. Note that there is an unequivocal discrimination between affected and control individuals at the higher bleomycin concentration. Patients are identified by family number (first digit) and individual number (second digit).

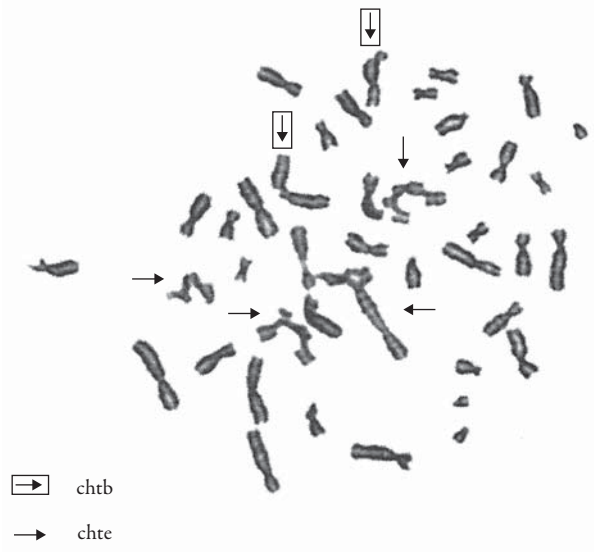

Figure 48.5 Lymphoblastoid cell line metaphase of a bleomycin-treated Nijmegen breakage syndrome cell showing chromatid breaks (chtb) and chromatid exchanges (chte).

ability following exposure to IR (Jaspers et al., 1988a; Taalman et al., 1983). Controversial results have been published concerning the hypersensitivity of NBS cells to alkylating agents. Seemanová et al. (1985) found weak evidence for increased chromosomal sensitivity to the bifunctional alkylating agent diepoxybutane (DEB) in one of two analyzed patients; Der Kaloustian et al. (1996) found normal DEB sensitivity in a third NBS patient. However, a clearly increased response to Trenimon was reported in siblings with NBS as early as 1991 (Wegner, 1991). Results obtained from these patients, now proven to carry the typical Slavic mutation 657del5, are shown in Figure 48.6. The speculation that the functionally deficient nibrin plays a central role in the aberrant repair processes of different types of DNA damage in NBS cells has been proven correct. Nakanishi et al. (2002) provided evidence for cooperation between the FA gene product FANCD2 and nibrin in the DNA cross-linking response (see Fanconi Anemia, above, and Molecular Interactions Between Nibrin and Other Proteins, below). Extensive studies of a cohort of NBS and a group of typical A-T patients corroborated the finding of marked sensitivity to the trifunctional alkylating agent Trenimon (Wegner et al., 1994). At doses of $10{\sim}^{8}$ M Trenimon, the chromosomal breakage rates of some NBS cell lines exceeded the control rate by more than 10 times (Stumm et al., 1997; Wegner, 1991; Wegner et al., 1994) (Fig. 48.6). A moderate sensitivity to MMC has been observed in one NBS patient (Chrzanowska et al., 2001). This moderate sensitivity to MMC is lower than in FA patients but has also been found in cell lines from other NBS patients (Kraakman-van der Zwet et al., 1999; Nakanishi et al., 2002; M. Stumm and R. Wegner, unpublished observation). These findings are of significance with respect to chemotherapy for NBS patients: the intensity of the treatment should be adjusted to the patient's individual risk factors and tolerance (Seidemann et al., 2000). Therefore, testing cells for their response to cytostatic agents should be considered and might reduce the risk of therapeutic side effects in sensitive patients.

Cell Cycle

Although the function of nibrin in cell-cycle control is far from fully understood, its involvement in cell-cycle checkpoints is supported by cellular, molecular, and cytogenetic data. Unlike in normal cells, DNA synthesis after IR or bleomycin treatment is not inhibited in NBS cells (Chrzanowska et al., 1995a; Jaspers et al., 1988a; Wegner et al., 1988). This feature, called *radioresistant DNA synthesis* (RDS), was initially described in A-T cells (Cramer and Painter, 1981; de Wit et al., 1981). RDS in NBS cells seems to be linked to a pathway involving the interaction of nibrin and SMC1. The inhibition of DNA synthesis after IR seems to depend on

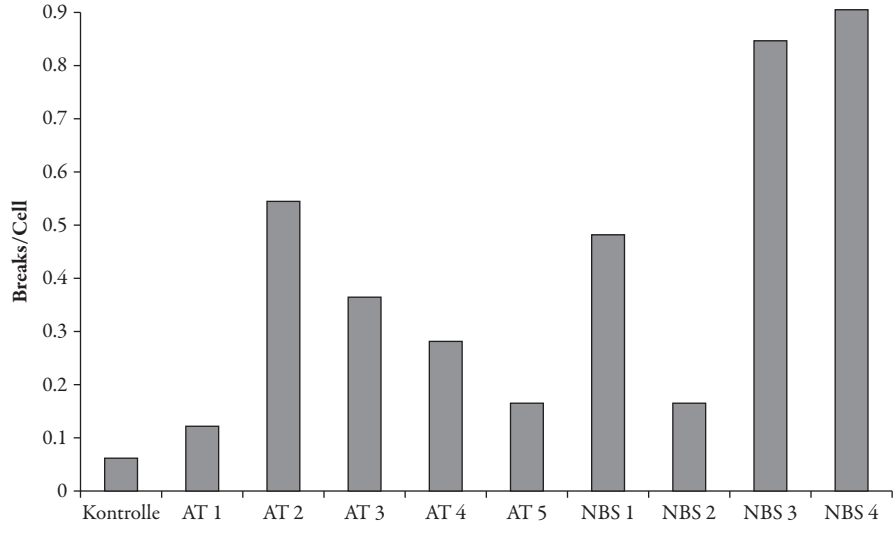

Figure 48.6 Trenimon-induced chromosomal breakage in lymphoblastoid cell line metaphases from Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia (AT) patients. Trenimon exposure (10−8 M) lasted 24 hours. At least 50 cells/column were analyzed.

phosphorylation of SMC1, a member of the cohesion proteins. Efficient phosphorylation of SMC1 by ATM requires nibrin (reviewed by Digweed and Sperling, 2004). In addition to this highly characteristic defect in an S-phase–dependent cell-cycle checkpoint, there are further disturbances in cell-cycle progression in some NBS cell lines. The role of *NBS1* in G1/S checkpoint control is controversial, because the extent of p53 accumulation and strength of expression of G1/S arrest after irradiation differ substantially in individual NBS cell lines (Antoccia et al., 1999; Girard et al., 2000; Jongmans et al., 1997; Matsuura et al., 1998; Sullivan et al., 1997; Yamazaki et al., 1998). An explanation for these variations might be dose-dependent differences in the response, as Girard et al. (2002) showed G1/S arrest in NBS cells at 1 Gy that was abolished at 5 Gy. Irrespective of differences in p53 induction and G1/S checkpoint control, NBS and A-T cells show comparable chromosome aberrations, both in quantity and in quality. This means that there is no simple correlation between checkpoint control and radiation-induced chromosomal instability. Therefore, factors other than those related directly to G1/S cell-cycle checkpoint control seem to be involved mainly in radiation sensitivity and chromosomal instability in NBS cells (Antoccia et al., 1999, 2002). The role of NBS 1 in G2 checkpoint control is also controversial, because the G2/M-phase transition appears to be extremely variable among different NBS cell lines. Some NBS cells, like A-T cells, fail to stop entry into mitosis after irradiation, a finding suggesting that NBS1 deficiency may disturb the G2/M checkpoint (Buscemi et al., 2001; Maraschio et al., 2001; Pincheira et al., 1998). In contrast, other NBS cells show a normal G2/M transition (Antoccia et al., 1997, 2002; Girard et al., 2000; Yamazaki et al., 1998). Because recent reports suggest an impaired ability of NBS cell lines to phosphorylate checkpoint kinase 2 (CHK2) (Buscemi et al., 2001; Girard et al., 2002), it is reasonable to expect effects of nibrin mutations on G2/M checkpoint control (see Molecular Interactions Between Nibrin and Other Proteins, below).

The expression of checkpoint impairment in irradiated NBS cells appears to vary considerably and be dose-dependent. Therefore, checkpoint disturbance is likely not the main reason for the strong radiation sensitivity in these cells over a broad range of doses.

GENETICS

Identification of the *NBS1* Gene

Despite clear differences in the clinical characteristics of NBS and A-T, cells from patients with these diseases express nearly identical cytogenetic and cellular features. This phenomenon led to the suggestion that NBS and A-T are two disorders due to allelic mutations (Curry et al., 1989). In contrast to this proposal, linkage analysis in six NBS families, formerly called ataxia-telangiectasia variant (AT-V) families, with at least two affected siblings proved unequivocally that the underlying *NBS* gene was not localized in the *ATM* region at 11q23.1 (Stumm et al., 1995). In agreement with this linkage exclusion, chromosome 11 could not complement the NBS phenotype on the cellular level in chromosome transfer studies (Komatsu et al., 1996). In 1988 it was postulated that NBS is a heterogeneous disease and that NBS patients fall into two complementation groups, AT-V1 (Nijmegen breakage syndrome) and AT-V2 (Berlin breakage syndrome) (Jaspers et al., 1988a, 1988b; Wegner et al., 1988). This conclusion was based on complementation studies using differentially labeled parental fibroblast cell lines in fusion experiments and comparing the rate of DNA synthesis in homokaryons with that in heterokaryons after irradiation or bleomycin treatment. Doubts about the reliability of this assay came with the report that A-T patients assigned to four complementation groups by employing the same assay actually all carried allelic mutations in the same gene, the *ATM* gene (Savitsky et al., 1995). Subsequent cell-fusion studies between AT-V1 and AT-V2 LCLs failed to achieve complementation, although there was clear normalization in the rate of chromosomal aberrations after fusion with normal cells (Stumm et al., 1997).

Finally, mapping of the *NBS* gene was initiated using two approaches. One was a whole-genome scan by polymerase chain reaction (PCR)-based micro satellite markers; the other followed the concept of unmasking heterozygosity. The latter study involved linkage studies in NBS families with markers spanning the potential gene loci at the breakpoints of a constitutional translocation t(3; 7)(q12; q31.3) in a NBS patient, where the derivative chromosomes were transmitted from the unaffected father to the affected daughter. Both linkage and haplotype analysis with markers spanning both translocation breakpoints on chromosomes 3 and 7 in 12 NBS families allowed the clear exclusion of an AT-V gene from the translocation breakpoints (Chrzanowska et al., 1997) but failed to identify the gene location. In contrast, the whole-genome scan was successful in localizing the gene. The analysis of 20 NBS patients from 14 families proved linkage to a 1 cM interval between markers D8S171 and D8S170 on chromosome 8q21 in all families. Furthermore, these results indicate that there is only one single *NBS* gene (Saar et al., 1997).

The *NBS 1* Gene

The gene underlying NBS, designated *NBS1,* was identified independently by two groups. Varon et al. (1998) employed the positional cloning strategy, whereas Carney et al. (1998) studied the hMre11/hRad50 double-strand repair complex and identified a third member of this complex, p95, as being the product of the *NBS1* gene, now called *nibrin.* As shown in Figure 48.7, the *NBS* gene spans about 50 kb of genomic DNA, has 16 exons, codes for a protein with 754 amino acids, and has a calculated molecular weight of 85 kDa. The mRNA is polyadenylated at two alternative sites, leading to mRNAs of 4.4 and 2.4 kb in all tissues examined thus far. Altogether, at least nine different mutations have been identified so far in NBS patients (Digweed and Sperling, 2004; Maraschio et al., 2001; Resnick et al., 2002; Varon et al., 1998; P. J. Concannon and R. A. Gatti, www.geneclinics.org). The 657del5 mutation is the most widespread Slavic founder mutation, which results in a frameshift causing the largest truncation of nibrin. Seven additional truncation mutations were identified in exons 6, 7,

8, and 10, all downstream of the forkhead-associated (FHA) and breast cancer carboxy-terminal (BRCT) protein domains (Fig. 48.7). Therefore, all NBS patients studied to date have large sequence losses in the C-terminal portion of the protein. Nevertheless, all patients express a truncated protein containing the N-terminal domains, a finding suggesting that these sequences may be essential during embryonic development and that *NBS1* null mutants would result in fetal death. In 2001, Maser et al. reported that NBS cells with the common 657del5 mutation produce a 70 kDa protein fragment ($NBS1p^{70}$) lacking the native N terminus, which associates with the hRAD50/hMRE11 complex. This NBS $1p^{70}$ fragment is produced by internal translation initiation within the *NBS1* mRNA using an open reading frame generated by the 657del5 frameshift. Therefore, the common *NBS1* 657del5 mutation seems to be a hypomorphic mutation, encoding a partially functional protein that diminishes the severity of the NBS phenotype. This hypomorphic mutation might be essential for fetal development in humans and other mammals, because null mutations have not been found in humans, and mouse null mutants are embryonic lethal (Dumon-Jones et al., 2003; Zhu et al., 2001). Interestingly, null mutations in *MRE11* and *RAD50* are also lethal in mammals (Luo et al., 1999; Xiao and Weaver, 1997). Although the amino acid sequence of nibrin shows no global homology to other known proteins, there are two domains within the N-terminal 200 residues that can be found in other proteins: a BRCT domain, first described in the *BRCA1* gene, and an FHA domain, named after the transcription factor family in which it was initially found (Fig. 48.7). Both of these domains are expected to be involved in protein–protein interaction. Two members of the NHEJ repair pathway of DNA DSBs, hMRE11 and hRAD50, were identified as molecular partners of nibrin (Carney et al., 1998). Nibrin shows a weak similarity (46 percent) to the yeast protein XRS2, restricted only to the first 115 amino acids. In contrast, the entire amino acid sequences of the MRE11 and RAD50 proteins are widely conserved from yeast to mammals.

MOLECULAR BIOLOGY OF NIBRIN

Nibrin appears to exert multifunctional activity. The identification of nibrin as a member of the human NHEJ repair complex of DSB suggests that defective DSB repair may be the central problem in NBS. The impasse involving p53 activation and cell-cycle arrest indicate that nibrin may also be involved in signal transduction. Another possible role of nibrin involves its interaction with histone γ-H2AX, forming a complex that associates with irradiation-induced DSBs (Kobayashi et al., 2002). However, nibrin seems to also have an important role in the direct processing of DSB. Within 30 minutes of irradiation, discrete nuclear foci can be visualized in the irradiated nuclei of cells by staining with antibodies to the repair proteins hMRE11 or hRAD50. Staining with antibodies to nibrin produces exactly the same pattern, demonstrating that nibrin remains in the nuclei for more than 8 hours after irradiation. The formation of nuclear foci is fully abrogated in NBS cells (Color Plate 48.II). Even the diffuse nuclear staining found in unirradiated normal cells is lacking

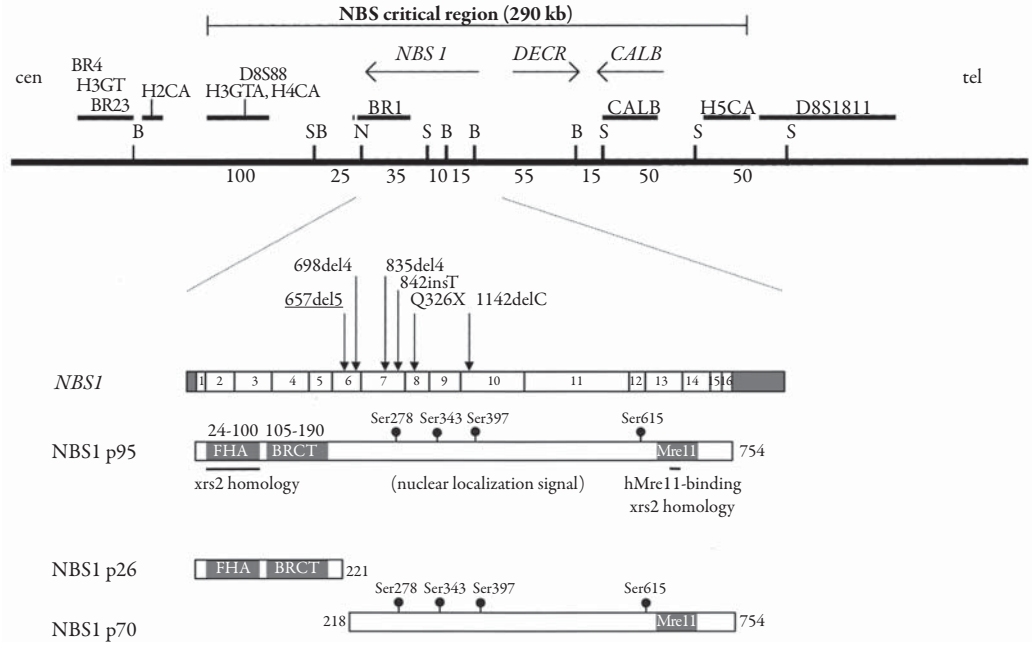

Figure 48.7 The *NBS1* gene and protein(s). Shown here is a physical map of the NBS1 region as defined by haplotype analyses of Nijmegen breakage syndrome (NBS) patients of Slavic origin. The positions of genes and genetic markers are indicated. The numbers below the line representing the DNA indicate the distances in kb between restriction enzyme sites (S, SalI; N, NotI; B, BamHI). Below the physical map, the exon structure of the *NBS1* gene is shown expanded. The position of mutations and the location of forkhead-associated (FHA) and breast cancer carboxy- terminal (BRCT) protein domains within the 754 residue p95 sequence are indicated. Serine residues that are phosphorylation targets are shown together with regions demonstrating similarity to yeast Xrs2 or implicated in binding to MRE11. Two protein fragments, p26 and p70, are found in NBS patient cells carrying the 657del5 mutation because of premature termination of translation and alternative translation, respectively.

in NBS cells, and considerable proportions of hRAD50 and hMRE11 are localized in the cytoplasm rather than in the nucleus. Since hRAD50 and hMRE11 are still found as a complex in NBS cells, a functional nibrin is clearly responsible for the location of these repair enzymes to the sites of DNA damage (Carney et al., 1998; Ito et al., 1999). In addition, nibrin is essential for the phosphorylation of hMRE11 after DNA damage (Dong et al., 1999), and it potentiates the ATP-driven DNA unwinding and endonuclease activity by the hRAD50/hMRE11 complex (Paull and Gellert, 1999). The hRAD50/hMRE11/nibrin complex has manganese-dependent, single-stranded DNA endonuclease and 3′ to 5′ exonculease activities; thus, it may be directly responsible for preparation of DNA ends for rejoining (Trujillo et al., 1998). Taken together, these findings indicate that nibrin plays an important regulatory role in the hRAD50/hMRE11/nibrin complex that responds to irradiation-induced DNA damage. The whole complex may act as a key factor in signaling and processing of DSB to initiate NHEJ and possibly homologous recombination as well.

Molecular Interactions Between Nibrin and Other Proteins

Considering the strong similarities in the cellular phenotypes of A-T and NBS, an important finding was the discovery that ATM and the hRAD50/hMRE11/nibrin complex function in the same network. Four laboratories reported that ATM modifies the hRAD50/hMRE11/nibrin complex by phosphorylation of nibrin (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000). Following exposure to IR, rapid phosphorylation of at least two serine residues of nibrin occurs. It is possible that the phosphorylation of distinct residues could modify the hRAD50/hMRE11/nibrin complex in different ways and therefore play a concerted regulatory role. This function is disturbed in NBS cells, because cells expressing mutant nibrin cannot be phosphorylated by ATM. ATM-dependent activation of CHK2, a key protein in cell-cycle control in response to DNA damage, requires functional nibrin (Buscemi et al., 2001). In addition to a role downstream of ATM, it is also now abundantly clear that the trimeric nibrin complex is involved in the effective activation of ATM itself. After DNA damage, ATM dimers dissociate and are autophosphorylated, for which the trimeric complex is required (Lee and Paull, 2004). Thus, apart from the function in DSB repair, the hRAD50/hMRE11/nibrin complex appears to be specifically required to activate checkpoint control pathways following the formation of DSBs (reviewed in D'Amours and Jackson, 2002).

Nibrin physically interacts with histone by direct binding to γ-H2AX, which is phosphorylated in response to the introduction of DSB and may therefore act as a very early responder to the DNA repair machinery. The FHA/BRCT domain of nibrin is essential for physical interaction with γ-H2AX and seems to play a crucial role in the binding to histone and in localization of the hMRE11/hRAD50 repair complex to damaged DNA (Kobayashi et al., 2002). The interaction may, however, be indirect since recent studies suggest binding of nibrin to the protein MDC1 via its FHA domain, with γ-H2AX then actually binding to MDC1 (Spycher et al., 2008). Additionally, as Nakanishi et al. (2002) were able to show, nibrin and the FA subtype D2 protein (FANCD2) cooperate in two distinct cellular functions, one involved in the response to DNA cross-links and another involved in the S-phase checkpoint control. Therefore, nibrin functions at the intersection of two main signaling pathways. In response to the DNA cross-linking agent MMC, nibrin assembles in subnuclear foci with hMRE11/hRAD50 and FANCD2. IR activates an S-phase checkpoint through the ATM- and NBS1-dependent phosphorylation of FANCD2. Thus, the involvement of nibrin in the pathway of DNA cross-linking repair, as shown by molecular and cellular analysis, fits with earlier cytogenetic results of a significant increase in chromosomal aberrations above normal levels in NBS cells exposed to Trenimon (Stumm et al., 1997; Wegner, 1991) or other cross-linkers as discussed above (in Cytogenetics).

Moreover, there is evidence that the hMRE complex also plays an important role in prevention of DSB formation during the normal replicative process. It has been shown that certain helicases, for example proteins mutated in Werner syndrome (Cheng et al., 2004) and in BS (Franchitto and Pichierri, 2002a, 2002b), associate with the hMRE complex via binding to NBS1 in vitro, and in vivo after IR or MMC exposure, preventing the accumulation of DSB during chromosomal DNA replication (Costanzo et al., 2001). These observations provide convincing evidence for a functional link of multiple proteins that are mutated in many of the known chromosomal instability syndromes, including FA, NBS, BS, Werner syndrome, and A-T (see Chapter 47).

HETEROGENEITY OF NBS

Some patients indistinguishable from NBS by clinical and cellular criteria do not carry mutations in the *NBS1* gene (Cerosaletti et al., 1998; Hiel et al., 2001, Maraschio et al., 2003). These observations strongly suggest mutations in one or more other genes that may cause NBS-like phenotypes. Indeed, a novel nonhomologous end-joining factor, NHEJ1, also called Cernunnos protein, has recently been identified and found to be mutated in patients with an NBS-like phenotype characterized by microcephaly, SCID, and chromosomal instability (Ahnesorg et al., 2006; Buck et al., 2006) (see Chapter 13).

PRENATAL DIAGNOSIS

Prenatal diagnosis of NBS is based on molecular methods whenever the underlying gene mutations in the index patient or parents are known. When no informative molecular data are available, RDS analysis in cultured chorionic villi or amniotic fluid cells is a reliable method for identifying NBS in a fetus (Der Kaloustian at al., 1996; Jaspers et al., 1990; Kleijer et al., 1994). Heterozygotes cannot be diagnosed by cytogenetic means (Wegner, 1991). Detailed protocols for cytogenetic analysis of NBS and other chromosomal instability syndromes are provided by Wegner and Stumm (1999).

TREATMENT AND PROGNOSIS

Traditionally, NBS patients are treated symptomatically. Intravenous administration of immunoglobulin (IVIG) and antibiotic prophylaxis may be an effective treatment in patients with significant immune deficiency and recurrent infections. One should also keep in mind that immunodeficiency mandates avoidance of live vaccines. Because cellular susceptibility to radiation and chemotherapy is increased in NBS patients, as in those with A-T, diagnostic irradiation should be reduced as much as possible and management of cancer therapy must be modified (Barth et al., 2003; Busch, 1994; Hart et al., 1987; Seidemann et al., 2000). NBS females presenting with hypergonadotropic hypogonadism require hormonal replacement therapy to induce and complete puberty and to avoid osteoporosis (Pozo and Argente, 2003). With symptomatic therapy alone, the overall prognosis for patients with NBS is unfavorable as compared to normal individuals, with premature death occurring due to either overwhelming infection or malignancies. A recent report describing the European experience with hematopoietic stem cell transplantation (HSCT) has reduced earlier concerns about increased toxicity of chemotherapy in NBS patients. Of the six patients transplanted for malignancy (n = 4) or severe immune deficiency (n = 2), five were alive and well after a median follow-up of 2.2 years (range: 1.7–8.1 years), with restored immunity. Donors included matched siblings (n = 2), matched unrelated donors (n = 2), and matched or mismatched family donors. The only fatality, due to sepsis, was a 16-year-old boy who was the only patient receiving myeloablative conditioning (Albert et al., 2010).

BLOOM SYNDROME

BS (MIM 210900) is an autosomal recessive chromosomal instability syndrome. The most consistent physical feature of BS is a strikingly small but well-proportioned body size and predisposition to the early development of a variety of cancers (German, 1993; German and Ellis, 2002). BS is the consequence of either homozygosity or compound heterozygosity of mutations in *BLM,* a gene that encodes the phylogenetically highly conserved nuclear RECQL2-helicase.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

BS was first recognized as a clinical entity by a dermatologist (Bloom, 1954). The most striking clinical feature, besides the small body size, is a sun-sensitive erythematous "butterfly" skin lesion of the face, a feature that diagnostically sets the smallness of BS apart from other growth deficiencies (Color Plate 48.III). The full spectrum of BS has been defined by collecting clinical data through the Bloom Syndrome Registry (German and Passarge, 1989), in which a cohort of 169 patients diagnosed before 1991 has been enrolled and a second cohort of 60 additional cases added after January 1991. The clinical features and complications that constitute the phenotype BS, based on the registry data, are summarized in Table 48.6 (German 1993; German and Ellis, 2002; unpublished registry data). Following Bloom's original report of a sun-sensitive "telangiectatic erythema resembling lupus erythematosus in dwarfs," additional classic features of BS were reported: (1) a striking sparsity of adipose tissue throughout infancy and childhood, (2) serious gastrointestinal problems during infancy, (3) susceptibility to respiratory tract and middle ear infections, and (4) sub- or infertility and an unusually early menopause. Nevertheless, at least five women with BS have become pregnant (Chisholm et al., 2001). Major complications of BS, subsequently observed and confirmed by findings from the registry, include chronic lung disease, which developed in at

Table 48.6 CLINICAL PHENOTYPE, IMMUNODEFICIENCY, AND CHROMOSOMAL ABNORMALITIES OF BLOOM SYNDROME

Growth and Development
Abnormally small body size
Sparseness of subcutaneous fat
Slightly disproportional microcephaly
Characteristic facial and head configuration
Minor anatomical malformations
Skin Lesions
Sun-sensitive erythema limited almost exclusively to the face and dorsa of hands and forearms
Non–sun-sensitive hyper- and hypopigmented areas
Gastrointestinal Symptoms
Most consistent during infancy
Gastroesophageal reflux, vomiting
Anorexia
Diarrhea
Infertility
Lack of sperm production
Premature menopause
Complications
Chronic lung disease
Diabetes mellitus, type II
Neoplasia
Immunodeficiency
Hypogammaglobulinemia
Depressed delayed type hypersensitivity
Susceptibility to infections, mostly bacterial
Upper and lower respiratory tract
Otitis media
Genomic Instability in Somatic Cells
Aberrant DNA replication
Increased chromosomal interchanges
Sister chromatid exchange
Quadriradials, telomeric association

least 9 of the 169 individuals from cohort I, causing premature death in 5 at a mean age of 24.6 years. Although considered to be a consequence of recurrent lung infections, an alternative etiology, gastroesophageal reflux, has been proposed as cause of the chronic lung pathology. Diabetes mellitus, diagnosed at a mean age of 25.7 years, and resembling adult-onset diabetes, developed in 31 individuals of cohort I. A total of 143 episodes of cancer have been diagnosed in 90 of the 169 patients with BS. This remarkable predisposition to the early development of cancer is a hallmark of BS (German et al., 1997) and of the other chromosomal instability syndromes described in this chapter and in Chapter 47. By age 25, approximately half of the patients with BS will have developed a malignancy, many of them more than one type. In the first two decades of life, the predominant types are leukemia and NHL. Later, carcinomas affecting predominantly the colon, skin, and breast are common. An increased risk of colorectal cancer in BLM heterozygosity has been observed in one study of Ashkenazi Jews (Gruber et al., 2002), but not in another (Cleary et al., 2003).

LABORATORY FINDINGS

Cytogenetics

Once BS is suspected, the diagnosis can be confirmed by karyotyping: the chromosomes of PHA-stimulated lymphocytes of an individual with BS exhibit an excessive number of SCEs (Fig. 48.8), a finding uniquely characteristic of BS (German et al., 1977).

In addition to the high numbers of SCEs, cultured B and T lymphocytes and skin fibroblasts from individuals with BS exhibit enhanced chromosome instability—"breakage"—as indicated by an increased number of chromatid gaps and breaks and structurally rearranged chromosomes. Approximately 1 percent of cultured lymphocytes in metaphase display a symmetrical four-armed configuration, designated as *quadriradial* (QR), composed of two homologous chromosomes. Opposite arms of the QR are of equal length, and the centromeres are positioned opposite one another (Fig. 48.8; see also Cytogenetics of FA, above; Fig. 48.1). A QR of the type characteristic of BS is cytogenetic evidence that during the preceding S phase an interchange took place between chromatids of the members of a homologous chromosome pair, and the point of exchange apparently was at homologous sites. Historically, QRs constituted the first evidence that homologous recombination can occur in mammalian somatic cells in the form of somatic crossing over.

Somatic Cell Mutability

Neither the SCEs nor the homologous chromatid interchanges exemplified by the QRs are necessarily mutagenic, although the interchanges would give rise to recombined chromosomes in half of the progeny of affected cells. However, the many gaps and breaks observed in metaphases do suggest that the excessive chromatid-exchange mechanism in BS cells is error-prone. Direct evidence of increased spontaneous mutability of

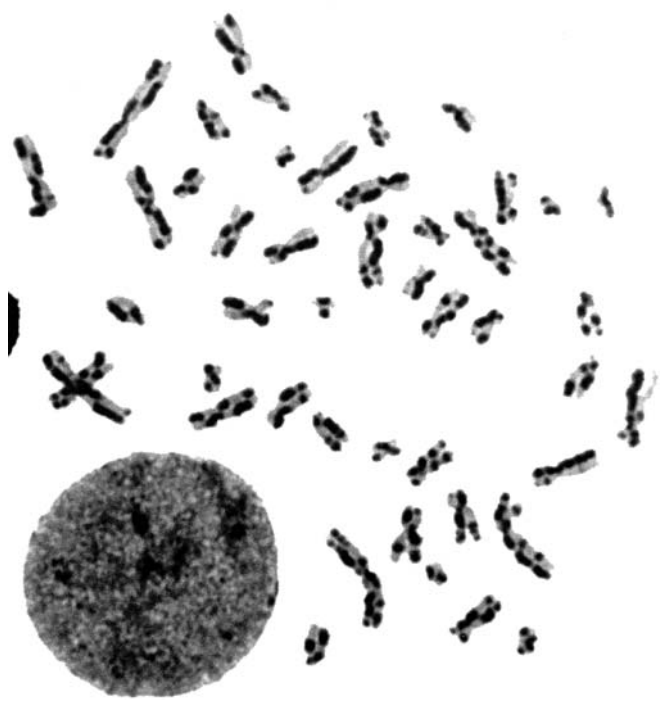

Figure 48.8 The characteristic and diagnostic elevation in number of sister chromatid exchanges (SCEs) in a cultured Bloom syndrome cell exposed to bromodeoxyuridine (BrdU) for two cell division cycles. Differentiation of the sister chromatids, dark or light, is accomplished by exposure of the BrdU-substituted chromosomes to the Hoechst dye 33258, then to light, and finally to Giemsa stain. The approximately 135 SCEs in this cell contrast with the fewer than 10 SCEs seen in similarly treated normal cells. A quadriradial chromosome is visible in the left field.

BS cells in vivo comes from studies that estimate the frequency of mutations affecting specific loci in human tissues. Coding loci that have been examined cytogenetically (but not at the molecular level) include the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) located on the X chromosome, the MN blood type (the glycophorin A locus on chromosome 4), and the major histocompatibility complex (on chromosome 6). For all three loci studied, the frequency of mutations in BS cells is greatly increased—for example, up to eight-fold for HGPRT (Vijayalaxmi et al., 1983) and 50- to 100-fold for the glycophorin A locus (Langlois et al., 1989). Finally, noncoding repetitive loci examined by molecular techniques were found to be hypermutatable, and molecular evidence confirming the cytogenetic evidence for excessive somatic recombination has been obtained in proliferating cells from a patient with BS (Groden et al., 1990). Thus, BS has to be considered a "mutator phenotype" (German, 1992) with mutations of many types, arising from nucleotide substitutions, deletions, and homologous recombination, and presumably affecting any part of the genome, accumulating excessively in proliferating cells both in vivo and in vitro.

Hematological Studies

Standard hematological parameters are normal except in patients who have developed leukemia or preleukemia.

Persistent anemia, usually mild and asymptomatic, is present in a subgroup of individuals with BS. Data submitted to the BS registry indicated that a myelodysplastic syndrome developed in 11 patients following cancer chemotherapy and spontaneously in one young-adult BS patient.

Endrocrine Studies

Ten individuals with BS, 9 months to 28 years of age, underwent endocrine evaluation; three nondiabetic children were found to have glucose intolerance and insulin resistance; two young adults had unsuspected diabetes or prediabetes. Growth hormone production was normal in all 10 patients studied (M. I. New, unpublished results).

Semen from approximately a dozen men with BS lacked spermatozoa completely with one exception, an adolescent who had sperm of abnormal morphology (J. German, unpublished results). Men heterozygous for a BS-causing mutation (fathers of BS patients) produced an increased number of spermatozoa with multiple breaks and rearrangements (Martin et al., 1994), suggesting that the infertility in men with BS is the consequence of a disturbance in meiosis. Supporting this summation is the finding that in normal mouse testes the BS protein (BSM) (vide infra) colocalizes with the replication protein A in the meiotic bivalent at the time of crossing over (Walpita et al., 1999).

Immunological Studies

Abnormal immune function, a common finding in BS patients, is highly variable and usually not severe (Etzioni et al., 1989; Hutteroth et al., 1975; Kondo et al., 1992a, 1992b; Taniguchi et al., 1982; Van Kerckhove et al., 1988; Weemaes et al., 1979, 1991). Hypogammaglobulinemia has been present in 87 percent of patients entered in the BS registry and involves one or more serum immunoglobulin classes (IgM, IgA, and, less commonly, IgG). Although most BS patients have decreased serum immunoglobulin levels, the majority have normal in vivo antibody responses to vaccines. Delayed-type hypersensitivity skin tests are generally negative, but skin reactivity to DNCB has been induced. Absolute lymphocyte counts and percentages of $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells are normal, and most BS patients have normal in vitro lymphocyte proliferation to mitogens. Abnormal NK-cell function has been reported (Ueno et al., 1985).

It is unclear if the increased rate of infection is a direct consequence of usually mild and variable immune deficiency. Data from the BS registry indicate that 86 percent of individuals who develop serious infections have a deficiency of one or more serum immunoglobulin isotypes, but in 14 percent immunoglobulin concentrations are normal. Of those patients without a history of infections, however, 28 percent have abnormally low immunoglobulin levels. V(D)J recombination (Hsieh et al., 1993) and somatic hypermutation of immunoglobulin genes (Sack et al., 1998) are normal and in agreement with the observation that most patients with BS respond normally to vaccinations.

GENETICS AND MOLECULAR BASIS OF BS

BS had been recognized throughout the world as a very rare genetic disease, with the exception of the Ashkenazi Jewish population, in which approximately 1 in 110 individuals is a carrier of BS. Genetic analysis is consistent with autosomal recessive transmission: parents of BS patients are clinically unaffected; both sexes are equally affected; the ratio of affected to nonaffected siblings among those born after the index case are approximately 1:4; and, in non-Ashkenazi families, parental consanguinity is over 25 percent. The high parental consanguinity rate among non-Ashkenazi families with BS combined with the hypermutability of BS cells allowed for homozygosity mapping to localize the gene to chromosome band 15q26.1 (German et al., 1994). The same group showed that 96 percent of patients whose parents were related were homozygous for a polymorphic marker in the pro-oncogene *FES,* strongly suggesting that the *BLM* gene is tightly linked to FES (Ellis et al., 1994). Ellis et al. (1995) observed that in some BS patients a minority of blood lymphocytes had normal SCE rates. Using lymphoblastoid cells established from patients with low SCE rates in peripheral blood lymphocytes, it was shown that polymorphic loci distal to the gene localization on 15q became homozygous, whereas markers proximal to the gene remained heterozygous. These findings were interpreted that low-SCE lymphocytes arose by recombination in patients who had inherited alleles from their (nonconsanguineous) parents that were mutated in different sites and thus had generated a functionally wild-type gene. On the basis of polymorphic markers used, the recombination must have occurred between the polymorphic loci D15S1108 and D15S127, a 250 kb region. Following the construction of a physical map of this region (Straughen et al., 1996), a candidate gene, designated *BLM,* was identified within this cDNA region (Ellis et al., 1995), and sequence analysis of this gene when using DNA from BS patients revealed nonsense and missense mutations. The BLM cDNA consists of 4,437 nucleotides and encodes a 1,417-amino acid protein with homology to the RecQ family of helicases in *Escherichia coli.* The finding that the high SCE rate observed in BS cells could be reduced to control values if a normal *BLM* gene was transfected into cells obtained from BS patients further confirmed that *BLM* was indeed the gene responsible for BS. BLM, therefore, is a member of the same subfamily of helicases that includes the Sgs1 protein of *Saccharomyces cerevisiae* and the human proteins WRN and RECQ4, mutated, respectively, in Werner syndrome and some cases of Rothmund-Thomson syndrome. The fact that *BLM* is most closely related to the yeast proteins Rghlp and Sgs (Kusano et al., 1999) is of interest because *BLM* has been demonstrated to suppress premature aging and increased homologous recombination caused by mutations of the *Sgs1* gene (Heo et al., 1999). Studies of mutants for *Dmblm*, the *Drosophila* homolog of human *BLM*, suggests that this gene functions in DSB repair (Adams et al., 2003; Kusano et al., 1999, 2001). BLM has been identified as a DNA binding protein with preference for DNA structures that resemble the presumed initial intermediate formed during homologous recombination; it can bind to and unwind such substrates in vitro, including holiday junctions and D loops (Van Brabant et al., 2000). These

observations, together with the high rate of QR and SCE formation, support the hypothesis that *BLM* plays an antirecombinogenic function and that cells from BS patients, in which *BLM* function is lacking, are hyperrecombinogenic.

Western blot analysis and immunofluorescence microscopy demonstrate that cultured lymphoblastoid cells from most BS patients lack BLM. In normal cells, BLM protein is localized within the nucleus, where its total amount, distribution, and colocalization with other nuclear proteins vary at different phases of the cell division cycle (Sanz et al., 2000).

BLM has been identified as a member of a group of proteins that associate with BRCA1 to form a large complex that has been named *BASC* (BRCA1-associated genome surveillance complex). This complex consists of tumor suppressors and DNA damage repair proteins including MSH2, MSH6, MLH1, ATM, BLM, and the RAD50/MRE11/NBS1 protein complex (Wang et al., 2000). These proteins colocalize to large nuclear foci when cells are treated with agents that interfere with DNA synthesis. All members of this complex play roles in the recognition of abnormal DNA structures or damaged DNA, suggesting that BASC may serve as a sensor for DNA damage. Thus, BLM is part of a protein complex that coordinates the activities required for maintenance and genome integrity during the process of DNA replication, activities suggesting a central role for BLM as well as NBS1 in DNA repair.

Transfection of a normal *BLM* gene into cultured SV40-transformed BS fibroblasts results in nuclear transfer of the BLM protein and reduction of the SCE rate, indicating that the helicase activity of BLM is required for its function (Ellis et al., 1999; Neff et al., 1999). The most common mutation of BLM is a 6 bp deletion/7 bp insertion in exon 10, which is the homozygous mutation causing BS in Ashkenazi Jews (Ellis et al., 1995). More than 60 unique mutations in the *BLM* gene have been identified in families with at least one affected individual. Homozygosity for a given BS mutation is found in most BS patients whose parents are known to be cousins. In non-Ashkenazi families, in which the parents are not related, compound heterozygosity is most common. There is no obvious genotype–phenotype correlation.

BLM is part of an evolutionarily conserved protein complex together with topoisomerase IIIα and other factors, including RMI1 and RMI2 (BTR complex). This BTR complex localizes to anaphase bridges during mitosis, and BS cells show excessive anaphase bridging. Some particularly fine bridges contained FANCD2 and FANCI and were enriched at fragile sites, where gaps, breaks, and chromosomal rearrangements cluster after replication inhibition. These findings suggest that incomplete replication at such sites require dissolution by the BTR complex during mitosis (Chan et al., 2009).

Other human genes in the RecQ family include *RECQ4,* mutated in Rothmund-Thomson syndrome (Lindor et al., 2000); *RECQ3* (WRN), mutated in Werner syndrome (Yu et al., 1996); and *RECQL/RecQ1* and *RECQ5*, for which no syndromes have yet been identified.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

Carrier females can be identified by DNA analysis if the mutation affecting the index case is known. A rapid method for detection of the Ashkenazi Jewish mutation is available (Straughen et al., 1998). Prenatal diagnosis of BS has been achieved by identifying a high rate of SCE in chorionic villi (Howell and Davies, 1994). Mutation analysis of fetal DNA is, however, the most reliable technique to establish the status of a fetus.

TREATMENT AND PROGNOSIS

Adequate nutrition is a common problem in infants and young children with BS, who typically show little interest in eating. This is compounded by frequent episodes of vomiting and diarrhea that may lead to life-threatening episodes of dehydration requiring hospitalization and may ultimately lead to the need for gastrostomy tube feeding. Infections require prompt diagnosis and adequate treatment with antibiotics. If hypogammaglobulinemia is documented and antibody deficiency demonstrated, the use of IVIG is recommended. In BS, as in patients with A-T, the extent of immune deficiency varies greatly.

Steps should be taken to protect the face of BS patients from sun exposure, especially during the early years of life. The diabetes mellitus observed in individuals with BS is not well characterized but resembles that of the adult-onset (type 2) condition, although it generally occurs earlier, usually in the 20s and 30s. Importantly, patients and their families should be informed about the high risk of developing cancer so that appropriate surveillance mechanisms can be put into place. Because individuals with BS are hypersensitive to chemotherapeutic agents, use of these compounds may have to be adjusted. Interestingly, the tumors of BS patients seem to be more sensitive to these therapeutic agents and cures have been reported using approximately half the dose of standard protocols.

Measures to increase height in patients, including growth hormone administration, have been largely unsuccessful. It has been postulated that the absence of BLM from somatic cells places a restriction on cell number and size, possibly through the deregulation of the p53 tumor suppressor pathway, which may not be limited to malignant cells (Garkavtsev et al., 2001).

ANIMAL MODELS

Disrupting *Blm* gene function in mice has created useful models for BS. Depending on the defect created by a targeted mutation of *Blm,* homozygous mutant animals may exhibit embryonic lethality, while in other mice homozygosity may yield viable and fertile animals with a cancer predisposition. In one model, a site upstream of the Blm helicase homology domain was targeted for disruption by use of homologous recombination. Whereas heterozygous mice appear normal and are phenotypically identical to wild-type littermates, mouse embryos homozygous for this mutation appear developmentally delayed and die by embryonic day 13.5 (Chester et al., 1998). $Blm^{-/-}$ littermates were smaller in size by 50 percent at 9.5 days postconception and remained smaller until death at 13.5 days postconception. Interestingly, the $Blm^{-/-}$ embryos were normal in their own body proportion and without structural abnormalities.

This BS knockout mouse model recapitulates many aspects of the human disease, including small size of the embryos, increased numbers of micronuclei in embryo blood cells, slow growth of cultured embryo fibroblasts, and, finally, a high number of SCE in cultured embryo fibroblasts, characteristically also seen in cells from Bloom patients. These data suggest that mutation of *BLM* in humans is sufficient to cause most aspects of the human disorder.

In addition, studies of mouse embryos clearly demonstrate increased programmed cell death, an observation not previously described in BS patients. There was also a marked reduction of the blood volume and the number of circulating red blood cells beginning at 9.5 days postconception; the affected embryos had only 5 or 10 percent as much blood as their wild-type littermates. Furthermore, an increased number of macrocytes and an increased presence of nuclear fragments were present in mutant versus wild-type erythrocytes (Chester et al., 1998).

Another knockout mouse model with a hypomorphic Blm protein produced a diminished quantity of normal mRNA and protein. These animals were viable and showed an inverse correlation between the quantity of Blm protein and the level of chromosome instability and a similar genotypic relationship for tumor predisposition, suggesting that Blm protein is rate-limiting for maintaining genome stability and the avoidance of tumors (McDaniel et al., 2003). These viable knockout mice had an 18-fold increase in the rate of somatic loss of heterozygosity (LOH), indicating a marked elevation of the mitotic recombination rate in mutated cells (Luo et al., 2000). Cell lines from these mice show elevations in the rate of mitotic recombination. These mice are also prone to a wide variety of cancers.

A unique mouse model was generated using gene targeting by homologous recombination to disrupt the mouse *Blm* gene to simulate BLM^{Ash}, a BS-causing mutant allele of BLM carried by approximately 1 percent of Ashkenazi Jews (Li et al., 1998). This mutation causes a frameshift in exon 10 that results in premature translation termination. Homozygous disruption of Blm by this method resulted in embryonic lethality. Cell lysates from heterozygous animals ($Blm^{+/-}$) had an approximately 50 percent reduction in Blm compared to wild type. There was a twofold increase in the number of micronuclei in BrdU-treated cultures of heterozygous deficient cells, a result suggesting that heterozygous cells have a subtle increase in genomic instability, presumably related to the reduced BLM level. There was a slight acceleration in the development of murine leukemia virus-induced metastatic T-cell lymphoma and an increased incidence of intestinal adenomas in mice heterozygous for this Blm mutation (Goss et al., 2002). These results demonstrate that Blm haploinsufficiency is sufficient to affect tumor formation in susceptible mice and probably to alter genome stability. This increased susceptibility to cancer in heterozygous mice supports the finding in human carriers for BS that they have an increased risk of colorectal cancer (Gruber et al., 2002).

Rare Syndromes with Chromosomal Instability

Patients with the clinical and cytogenetic features of chromosomal instability syndromes, in particular NBS and AT, but without mutations in the respective genes are of particular interest because they may disclose clues about unknown gene products involved in the network of genome integrity surveillance. Ligase I (LIG1) deficiency (MIM 126391) was described in one female patient with typical features of a chromosomal instability syndrome—growth retardation, telangiectasia, immunodeficiency, and lymphoma—at the age of 19 years (Webster et al., 1992). Hypersensitivity to UV light, alkylating agents, and IR was observed at the clinical and cellular levels. Molecular analysis identified two missense mutations in the *LIG1* gene of this patient (Barnes et al., 1992). A link to the NHEJ repair network was suggested by the finding of a physical association of LIG1 with MRE11 (Petrini et al., 1995). Only one patient with LIG1 deficiency has been described thus far. This low number might be explained by the minor role of LIG1 in NHEJ compared to that of ligase IV (LIG4) (see below), which can be substituted more easily. Another explanation might be the presence of undetected mutations in another gene involved in NHEJ.

More recently, mutations in the *LIG4* gene were shown to cause an NBS-like syndrome in four patients (O'Driscoll et al., 2001) (Table 48.1, see Chapter 13). On the cellular level, cell lines from LIG4-deficient patients show pronounced radiosensitivity similar to that in NBS. In contrast to NBS cells, LIG4-deficient cells show normal cell-cycle checkpoint responses but impaired DSB rejoining. Since V(D)J recombination is also affected, immunodeficiency is part of the clinical phenotype. Thus, LIG4, if mutated, is responsible for the defective DSB repair, resulting in a novel combined immunodeficiency syndrome (LIG4 deficiency syndrome, MIM *606593). By targeted disruption of both *LIG4* alleles in a human pre-B-cell line studied for radiosensitivity, Grawunder et al. (1998) showed that LIG4 activity is responsible for the ligation step in nonhomologous DNA end-joining and in V(D)J recombination.

It has been demonstrated that LIG4 forms a tight complex with the protein XRCC4 (Grawunder et al. 1998) to repair DSB by the NHEJ pathway. However, it took 8 more years to identify the third partner of this complex, which was suspected by Dai et al. (2003) while studying a patient with T-B-SCID whose cells showed dramatic radiosensitivity, decreased DSB rejoining, and reduced fidelity in signal and coding joint formation during V(D)J recombination. This patient (2BN), while sharing several characteristics of NBS and of LIG4 deficiency, did not have mutations in the respective genes (Dai et al., 2003). Two groups recently reported independently mutations in a hitherto unknown gene through cDNA functional complementation cloning (Buck et al., 2006) and yeast two-hybrid screening for *XRCC4* interactors (Ahnesorg et al., 2006), respectively. This novel protein has been named Cernunnos (Buck et al., 2006) or XLF (Ahnesorg et al., 2006) and was officially designated as nonhomologous end-joining factor 1 (NHEJ1). Mutations in this gene are responsible for the clinical phenotype mentioned above, including microcephaly, and for defective NHEJ and V(D)J recombination (MIM 611291) resulting in severe combined immune deficiency (Buck et al., 2006) (see Chapter 13).

Waltes et al. described a patient with a disorder resembling NBS who harbored different hypomorphic mutations,

one in each allele of the *RAD50* gene (Waltes et al., 2009). The girl, originally described in 1991 by Barbi et al., presented with many clinical characteristics of NBS, but not with immunodeficiency (Table 48.1). The Rad50 deficiency (MIM *604040) resulted in increased spontaneous chromosomal instability, radiosensitivity, RDS, impaired nibrin phosphorylation, and impaired nuclear foci formation after irradiation. Since hRAD50 is one of the three partners of the hMRE11/hRAD50/nibrin complex, it is not surprising to find the NBS phenotype in a compound heterozygous individual. The link between RAD50 deficiency and increased radiosensitivity points to the importance of a fully functional hMRE11/hRAD50/nibrin complex for reliable NHEJ.

More surprising is the clinical picture of patients carrying mutations in the *hMRE11* gene (Stewart et al., 1999) (Table 48.1). Because these patients show many similarities to A-T, although with a milder phenotype and without mutations in the *ATM* gene, this syndrome has been called ataxia-telangiectasia–like disorder (A-TLD) (MIM 604391). The protein hMRE11 is engaged in forming the hMRE11/hRAD50/nibrin complex, which plays an early role in a signal transduction pathway responding to DSB induced by IR and radiomimetic chemicals (Taylor et al., 2004). Both these events activate DNA repair as well as cell-cycle checkpoint controls. All *hMRE11* mutations observed in this recessive disorder result in the expression of proteins with some residual function that may be necessary for the survival of affected patients. Typically, this residual function results from a hypomorphic mutation of at least one *hMRE11* allele.

A recently described DNA repair defect, Riddle syndrome (MIM 611943), characterized by mild motor control and learning difficulties, facial dysmorphism, short stature, immunodeficiency and radiosensitivity, is caused by mutations in RNF168, a ubiquitin ligase. RNF168 is recruited to the site of DNA damage by binding to ubiquitylated histone H2A, resulting in chromatin modification and accumulation of 53BP1 and BRACA1 to DNA lesions (Stewart et al., 2009).

Immunodeficiency with centromere instability and facial anomalies (ICF syndrome) (MIM 242860) is reported in Chapter 49.

CONCLUDING REMARKS

The descriptive level of chromosomal instability syndromes of only one decade ago has been complemented by the discovery that the chromosomal instability syndromes not only form subgroups based on their descriptive features but are actually linked together at the molecular level. Most if not all genes mutated in the syndromes described in this chapter (including *ATM,* see Chapter 47) take part in the complex network of DSB sensing, signal transduction, and DNA repair. Although much more has to be done to determine specific functional aspects, the facts known to date allow a first glimpse into the complex network of genomic stability maintenance. A very simplified schema of this network is shown in Figure 48.9, which lists only those gene products mutated in the chromosomal instability syndromes presented in this chapter. Their physiological functions have been demonstrated by the analysis of gene mutations in relevant patients. The identification and investigation of additional pathological conditions

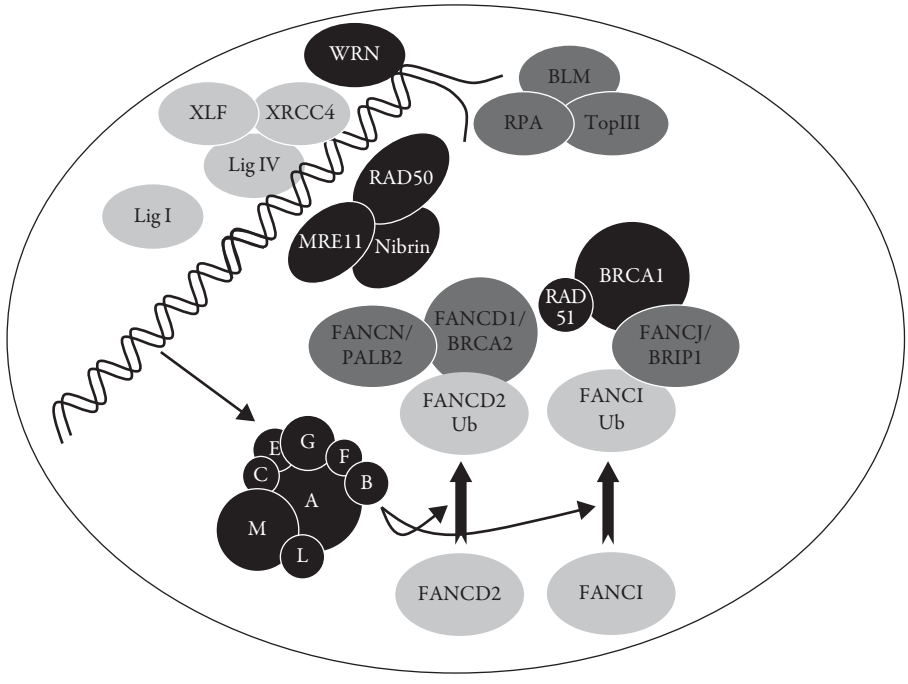

Figure 48.9 The network of proteins mutated in chromosomal instability syndromes. This highly simplified schematic drawing shows the proteins acting in the DSB repair network. Only proteins mutated in the chromosomal instability syndromes discussed in this chapter are shown. Several protein complexes can be identified: the FA core complex, the MRE11/RAD50/nibrin complex, the LIG4 complex, the BLM complex, and the complex consisting of BRCA1/BRAC2/RAD51/FANCD2/FANCI. The BASC complex (Wang et al., 2000) includes BRCA1, BLM, and RAD50/MRE11/nibrin. Lig, ligase; WRN, Werner syndrome; XLF, XLF/NHEJ1/Cernunnos protein.

are essential to a better understanding of these crucial cellular processes; for the benefit of our patients, it is even more important to generate new ideas that lead to more effective treatments of these devastating genetic disorders.

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49.

IMMUNODEFICIENCY WITH CENTROMERE INSTABILITY AND FACIAL ANOMALIES (ICF SYNDROME)

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Immunodeficiency with centromeric instability and facial anomalies (ICF syndrome) (MIM #242860) is a rare autosomal recessive disease characterized by immunodeficiency of variable extent, mild facial anomalies, and chromosome instability involving the pericentromeric regions of chromosomes 1, 9, and 16. Two groups independently reported the disease in the late 1970s (Hultén, 1978; Tiepolo et al., 1978, 1979). A patient described earlier by Østergaard (1973) also appears to have had ICF syndrome based on the clinical symptoms and the chromosomal abnormalities reported. The acronym ICF was suggested in accordance with the characteristic features of the syndrome: *I*mmunodeficiency, *C*entromeric instability, and *F*acial anomalies (Maraschio et al., 1988). Only about 50 ICF patients have been described in the literature thus far, but underdiagnosis of the syndrome is quite possible because of phenotypic variability and infrequent testing for the diagnostic chromosome anomalies. Here we summarize the common clinical features of this limited set of patients as well as the molecular defects associated with ICF.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Although ICF patients have been found all over the world, they are concentrated in the Mediterranean area (de Greef et al., 2011; Hagleitner et al., 2008). Inheritance is autosomal recessive and consanguinity has been found in several ICF families. At least 20 of the known patients have died, most of them before the age of 12 years. The predominant cause of death was severe respiratory tract infections, sepsis, and malignancy (Hagleitner et al., 2008).

As described below, two genes known to be mutated in ICF are *DNMT3B* (ICF1) and *ZBTB24* (ICF2). ICF1 and ICF2 patients have a similar phenotypes (Hagleitner et al., 2008), including the same facial anomalies, lower respiratory tract infections, opportunistic infections, and immunodeficiency.

FACIAL ANOMALIES

Mild facial dysmorphic features are found in most ICF patients (Table 49.1). Hultén, in 1978, described a boy with a peculiar facial appearance characterized by bilateral epicanthus and slight protrusion of the right external ear (Hultén, 1978). The patient described by Tiepolo et al. had an epicanthus and low nasal bridge (Maraschio et al., 1988; Tiepolo et al., 1978, 1979). Several other dysmorphisms have been described subsequently, including macroglossia (Fryns et al., 1981), micrognathia (Howard et al., 1985), and hypertelorism (Carpenter et al., 1988).

Phenotypic characteristics currently considered part of ICF syndrome may include a round face with epicanthus, telecanthus, flat nasal bridge, hypertelorism, upturned nose, macroglossia, micrognathia, and low-set ears. Hypertelorism, flat nasal bridge, epicanthic folds, and low-set ears were most frequent. Many of these features become more prominent during later childhood; however, dysmorphic features are not always present in adult ICF patients. Thus, patients with agammaglobulinemia in whom B cells are present should be considered for cytogenetic analysis to preclude ICF syndrome.

Facial anomalies may or may not be present immediately after birth. For example, the brother of the patient described by Smeets et al. (1994) did not show dysmorphic features until the age of 1 year, when he presented with a round face and telecanthus (Color Plate 49.I).

GROWTH AND DEVELOPMENT AND NEUROLOGICAL PROBLEMS

Although some ICF patients were significantly smaller than normal at birth, most show normal birth weight. Growth

Table 49.1 CLINICAL FEATURES AND GENE MUTATIONS IN ICF SYNDROME

	TOTAL	*DNMT3B* MUTATION	*ZBTB24* MUTATION
Number of patients	52	24	7
Consanguinity		13	5
Died	19/48	10/24	3/7
Facial anomalies	45/47	20/21	7/7
Growth and development			
Birth weight <10th percentile	22/38	7/19	3/4
Growth <3rd percentile		9/14	
Failure to thrive	23/44	8/18	1/7
Delays in motor development	22/44	10/19	4/5
Delays in speech and language development	21/44	9/16	5/7
Intelligence			
Normal	17/44	11/20	
Mild retardation	17/44	4/20	7/7
Moderate retardation	10/44	5/20	
Gastrointestinal problems			
Diarrhea	20/44	7/18	2/7
Neurology			
Cerebral atrophy	7/50	4/24	
Seizures	5/50	3/22	0/7
Infection			
Bronchopneumonia	37/45	16/19	7/7
Otitis	15/45	9/19	2/7
Sepsis	10/46	4/20	1/7
Candida infection	9/37	4/20	2/7
Pneumocystis jirovecii	4/37	2/20	2/7
Immunology			
Agammaglobulinemia	28	15	5
Hypogammaglobulinemia	17	7	2
Selective IgM deficiency	2		
Selective IgA deficiency	1	1	
IgG2 subclass deficiency	1		
CD4 cells decreased	7/41	3/18	1/7
Lymphocyte stimulation in vitro decreased	5/30	2/14	

retardation occurred in some patients because of failure to thrive following infections or gastrointestinal problems. The majority of ICF patients have shown a delay in walking, as well as in initial speech. Additionally, hypotonia was demonstrated in several patients (Carpenter et al., 1988; Petit et al., 1997; Schuffenhauer et al., 1995). Some ICF children needed physiotherapy and/or speech therapy to learn to walk or talk.

The intelligence of ICF patients is quite variable. A few patients were moderately or severely mentally retarded (Gimelli et al. 1993; Howard et al. 1985; Schuffenhauer et al. 1995 Tiepolo et al. 1979). The initial cytogenetic analysis for some of these patients, however, was performed because they presented with mental retardation and dysmorphisms rather than immunodeficiency. One child was reported as doing exceptionally well in high school, showing that the disorder can exist without retardation. Children with ICF frequently have difficulty with speech and gross motor skills. Physiotherapeutic support and speech therapy often have a positive influence on their development. Hagleitner described five patients with cortical atrophy, two of whom developed generalized tonic-clonic seizures within their first 2 years of life. Two other patients had a progressive deterioration of cognitive functions at the age of 10 years (Hagleitner et al., 2008).

Because many patients succumb to infections at an early age, little is known about sexual development in ICF

individuals. Puberty generally appears to be normal in the few cases followed. A pregnancy has been reported, for example, in one adult female patient, although her older affected brother is azoospermic (G. Gimelli, personal communication).

The most common malformations found are inguinal hernia and hypospadias, cleft palate, and syndactyly (Hagleitner et al., 2008). Cardiac anomalies were reported in two patients and included ventricular septal defect and atrial septal defect. Congenital hypothyroidism was found in one patient. Cerebral malformations were reported in individual patients and include focal cortical heterotopy, corpus callosum hypoplasia, and hydrocephalus.

INFECTIONS

Infections are a prominent clinical feature in ICF syndrome, especially respiratory tract infections, which occur in nearly all cases (Table 49.1). Pneumonia, often caused by *Haemophilus influenzae* or *Streptococcus pneumoniae* (Howard et al., 1985; Østergaard, 1973) is a common affliction. Bacterial sepsis occurred in several patients due to infection with *Klebsiella*, *Pseudomonas*, or *Staphylococcus aureus*. In addition, opportunistic infections such as *Pneumocystis carinii (jiroveci)* pneumonia (PCP) (Bauld et al., 1991), cytomegalovirus (Fasth et al,. 1990), and *Candida albicans* (Fasth et al., 1990; Valkova et al., 1987) also frequently occur in ICF patients. One patient with selective IgM deficiency developed a progressive multifocal leukoencephalopathy due to JC virus at the age of 35 years; she concurrently had agammaglobulinemia (Colucci et al., 2004). Infections with JC virus, *P. jiroveci*, and *Candida* suggest that a subtle defect of T-cell function must be present, apart from the hypogammaglobulinemia. In some ICF patients a T-cell defect has been demonstrated (Table 49.1); however, one of the patients presenting with PCP seemed to have had only a B-cell deficiency (Bauld et al., 1991).

Despite substitution therapy with immunoglobulins, many ICF patients have died from infection at a very young age. Allogeneic stem cell transplantation has been performed successfully in four patients (Gennery et al., 2007).

GASTROINTESTINAL PROBLEMS

Protruding abdomen has been described in several ICF patients (Fryns et al., 1981; Smeets et al., 1994; Turleau et al., 1989). Failure to thrive is common. Many patients suffer diarrhea (Fasth et al., 1990; Maraschio et al., 1988; Smeets et al., 1994; Turleau et al., 1989; Valkova et al., 1987), and extended total parenteral feeding was necessary in several children (Turleau et al., 1989; Valkova et al., 1987). Microorganisms such as *Cryptosporidium* or *Giardia lamblia* were not found in cultures from these patients. Malabsorption was reported in several patients (Fryns et al., 1981; Petit et al., 1997; Smeets et al., 1994; Turleau et al., 1989), and villous atrophy has been demonstrated in some patients (Carpenter et al., 1988; Fryns et al., 1981; Turleau et al., 1989).

MALIGNANCIES AND HEMATOLOGICAL ABNORMALITIES

Aplastic anemia has been reported in ICF (Hagleitner et al., 2008). Acquired leukocytopenia and thrombocytopenia of unknown origin were documented in several patients. In two patients, cortical atrophy evolved at the same time without evidence for an infectious origin.

The observed progressive deterioration and/or thrombocytopenia and leukocytopenia in combination with cerebral atrophy is similar to the pathology of neurological manifestations in systemic lupus erythematosus, another disease linked to DNA hypomethylation (Sekigawa et al., 2006). Development of hematological abnormalities such as aplastic anemia or thrombocytopenia and leukocytopenia, hepatosplenomegaly, and arthritis may be caused by autoimmune mechanisms. Autoimmunity in the absence of antibodies suggests activation of autoreactive T cells by T-cell receptor or by innate activating receptors directly.

Hematological malignancies were reported in two patients and included one ICF2 patient who developed a classical Hodgkin's lymphoma (Schuetz et al., 2007) and one ICF1 patient who acquired a common pre-B leukemia. Additionally, a second ICF1 patient died from angiosarcoma (van den Brand et al., 2011).

A predisposition to cancer is frequently associated with immunodeficiency. Various types of cancer share with ICF syndrome cells the characteristic of hypomethylated satellite 2 DNA, which is known to be associated with chromosomal instability and is believed to promote tumor frequency and malignancy (Ueda et al., 2006).

LABORATORY FINDINGS

IMMUNOLOGICAL FINDINGS

One of the characteristic findings in the ICF syndrome is immunodeficiency (Table 49.1), which can be quite variable, ranging from severe immunodeficiency to a near-normal immune system. Agammaglobulinemia with normal B cells is the most common finding in ICF patients. Some patients have decreased levels of all immunoglobulins, and four patients were reported with selective IgA, IgM, or IgG2 subclass deficiency. One patient had only a slightly decreased number of CD4-positive cells (Gimelli et al., 1993). Selective IgM deficiency and failure to produce antibodies to polysaccharide antigens, but a normal response to protein antigens such as tetanus toxoid, was observed in a patient with ICF who was subsequently found to have normal IgM levels and normal isohemagglutinin titers; antibodies to *H. influenzae*, however, remained absent (Østergaard, 1973, 1977).

Normal percentages of T cells were observed in most patients tested, and CD4-positive cells were decreased (<10th percentile) only in some patients (Hagleitner et al., 2008). A limited number of patients underwent assessment of T-cell function in vitro. Five of 30 patients tested showed decreased or absent cell proliferation when stimulated with the mitogen PHA.

CYTOGENETIC ABNORMALITIES

Aberrations of chromosomes 1 and/or 16 involving their heterochromatic pericentromeric DNA sequences can be observed at high frequency in regular lymphocyte cultures of peripheral blood samples from ICF patients. These aberrations can be recognized as gaps, breaks, deletions, isochromosomes, multiradial figures, interchanges between pericentromeric regions, and multiradial configurations (Fig. 49.1). Two short arms of chromosomes 1 and 16 are present in all aberrant cells, with a varying number of long arms, suggesting that breaks occurred just below the centromere within the heterochromatic region on the proximal long arm (Haas, 1990). Similar aberrations involving chromosome 9 (Fryns et al., 1981; Tiepolo et al., 1979; Valkova et al., 1987) and, rarely, chromosomes 2 (Valkova et al., 1987) and 10 (Turleau et al., 1989) have also been reported.

The overall frequency of ICF cells with chromosomal aberrations often increases with culture time (Maraschio et al., 1988; Smeets et al., 1994; Tiepolo et al., 1979), although the opposite has also been reported (Turleau et al., 1989). Moreover, aberrations can also become more complex with increasing time in culture (Howard et al., 1985; Hultén, 1978; Smeets et al., 1994; Tiepolo et al., 1979). Interphase nuclei consistently show an increased number of micronuclei and/or nuclear protrusions (Fasth et al., 1990; Maraschio et al., 1992). Fluorescence in situ hybridization studies have clearly demonstrated that portions of chromosomes 1 and 16 are present in these micronuclei (Maraschio et al., 1992; Smeets et al., 1994). Cultured peripheral blood lymphocytes always display numerous aberrations, whereas other tissues, such as cultured fibroblasts, Epstein-Barr-virus–transformed B cells, and bone marrow cells, usually show many fewer, if any, aberrant cells. When aberrations are present in such cells, they are usually reduced in complexity compared to those seen in lymphocytes (Fasth et al., 1990; Gimelli et al., 1993; Howard et al., 1985; Maraschio et al., 1989; Turleau et al., 1989).

STRATEGIES FOR DIAGNOSIS

Without chromosome studies, most patients with ICF syndrome would be classified as suffering from common variable immunodeficiency (CVID) according to the WHO classification for primary immunodeficiencies (see Chapter 28). ICF syndrome should be considered, therefore, for all patients with B-cell–positive agammaglobulinemia or CVID without a known gene defect, even when facial anomalies are absent. A history of delayed milestones, such as walking and speech, is also suggestive for ICF syndrome. Diagnosis of ICF syndrome can be made by cytogenetic analysis and Southern blot analysis of satellite DNA methylation.

PRENATAL DIAGNOSIS

Prenatal diagnosis of ICF syndrome by mutation detection has been performed successfully in ICF1 patients (Rigolet et al., 2007). Now that the molecular defect for a large proportion of ICF2 patients has been identified as mutations in *ZBTB24* (as discussed below) (de Greef et al., 2011), prenatal diagnosis can be extended to include both genes *DNMT3B* and *ZBTB24*.

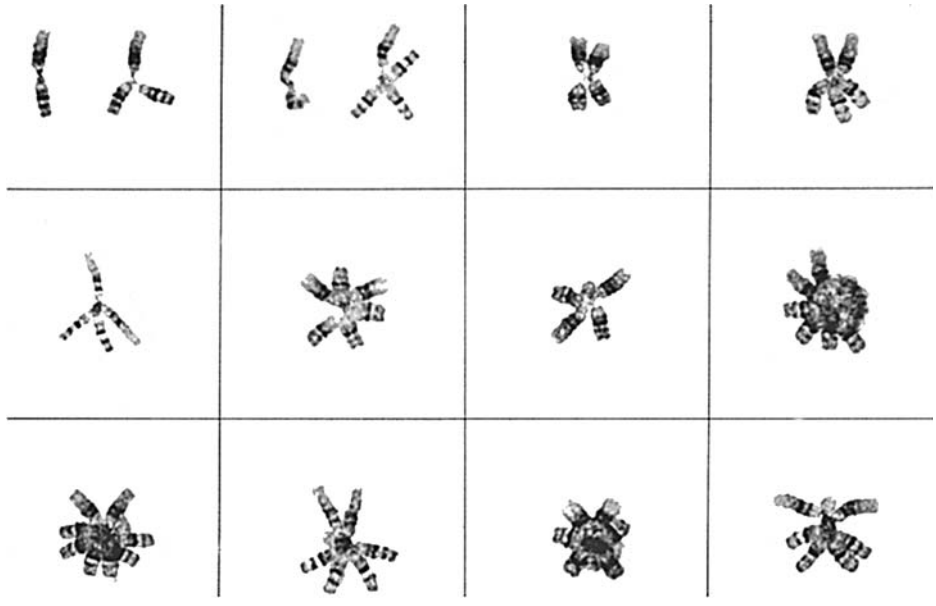

Figure 49.1 Top row: chromosome 1 abnormalities. A, Chromosome 1 with two long arms (down). B, One normal chromosome 1 and one chromosome 1 with three long arms. C, Two chromosomes 1 fused in the paracentric heterochromatin. D, Two chromosomes 1 fused with two short arms (up) and three long arms (down). Row 2: E, Two chromosomes 1 fused in the paracentric heterochromatin, F, Two chromosomes 1 fused with two short arms (left and right upwards) and four long arms. G, Two chromosomes 1 and a piece of chromosome 16 fused in the paracentric heterochromatin. H, A complex rearrangement of both chromosomes 1 and 16 with multiple long arms of both chromosomes. Row 3: I, A complex rearrangement of both chromosomes 1 and 16 with multiple long arms of both chromosomes; J. two short arms of chromosome 1 (up) and 4 long arms of chromosome 1 (down) with a piece of chromosome 16 centrally. K, A complex rearrangement of both chromosomes 1 and 16 with multiple long arms of both chromosomes. L, Two short arms of chromosome 1 (up) and 4 long arms of chromosome 1 (down) with a piece of chromosome 16 centrally.

TREATMENT AND PROGNOSIS

Nearly all ICF patients receive regular infusions of intravenous immunoglobulin (IVIG). This treatment reduces the severity and frequency of infections in most patients. Despite IVIG treatment, however, at least 15 patients died in infancy or in childhood because of infections (Fasth et al., 1990; Fryns et al., 1981; Valkova et al., 1987; C. Weemaes, unpublished observations). Most of these patients suffered from agammaglobulinemia with some T-cell defects, but a patient with only IgA deficiency also died at a young age (11 years) (Tiepolo et al., 1979).

Opportunistic infections with *P. jiroveci* resulting in interstitial pneumonia (PCP), cytomegalovirus, and *Candida* have been described (Bauld et al., 1991; Fasth et al., 1990; Valkova et al., 1987). Prophylactic use of trimethoprim-sulfamethoxazole to prevent PCP seems to be indicated, even when a T-cell defect has not been demonstrated.

Hematopoietic stem cell transplantation should be considered in patients suffering from infections and failure to thrive. It was successful in three such patients (Gennery et al., 2007). Because B cells are defective in ICF syndrome (Blanco-Betancourt et al., 2004), it is important to consider a myeloablative regimen in order to achieve reconstitution of both T and B lymphocytes. Further investigation using mouse models should help to evaluate the efficacy of potential therapies.

MOLECULAR BASIS: MUTATIONS IN *DNMT3B* AND *ZBTB24*

The extremely rare occurrence of the ICF syndrome and the existence of affected consanguineous individuals strongly suggested an autosomal recessive transmission and made the localization of the ICF gene a suitable target for homozygosity mapping. Wijmenga (Wijmenga et al., 1998) performed homozygosity mapping in three consanguineous ICF families: one of Dutch ancestry and two of Turkish ethnicity. This study identified a 9 cM region on chromosome 20q11-q13 that was homozygous in all affected inbred individuals.

Although the etiology of ICF syndrome was not clear at that time, the hypomethylation that is observed in ICF patients suggested a role for DNA methylation in this disorder (Jeanpierre et al., 1993). This hypothesis was further substantiated by the observation that the cytogenetic abnormalities in ICF syndrome could be complemented by fusion of ICF cells to normal cells (Schuffenhauer et al., 1995). Cell fusion also led to a partial restoration of the de novo methylation of ICF satellite 2 and 3 sequences (Hansen et al., 1999), presumably through the activity of a functional de novo DNA methyltransferase from the non-ICF cell. A search of the raw sequence data from human chromosome 20 for the presence of a DNA methyltransferase gene led to the identification of a gene highly homologous to murine *Dnmt3b*. The *DNMT3B* gene was localized to 20q11 within the 9 cM region to which the ICF gene was localized previously. Mutations of this gene in ICF patients confirmed that *DNMT3B* was indeed a gene responsible for ICF syndrome (Hansen et al., 1999; Okano et al., 1999; Xu et al., 1999).

As not all cases of ICF syndrome could be explained by mutations in *DNMT3B*, the search for ICF genes continued. Different from ICF1 patients, with mutations in *DNMT3B*, these *DNMT3B* mutation-negative patients, or ICF2 cases, all show hypomethylation of α-satellite repeat DNA on chromosome 9 in addition to those regions previously shown to be hypomethylated in ICF1 patients (Miniou et al., 1997). By combining homozygosity mapping in consanguineous ICF2 families with exome sequencing, loss-of-function mutations in *ZBTB24* were identified as the underlying cause of 60 to 70 percent of ICF2 patients (de Greef et al., 2011). As one consanguineous ICF patient was excluded for both loci, it is likely that there is at least one more locus involved in the development of ICF syndrome.

DNMT3B GENE STRUCTURE AND MUTATIONAL ANALYSIS

The *DNMT3B* gene spans a region of about 50 kb and encodes a transcript of 4.3 kb representing 23 exons, 22 of which are coding (Xie et al., 1999). Alternative splicing of the gene leads to at least five isoforms: *DNMT3B1*, *DNMT3B2*, *DNMT3B3*, *DNMT3B4*, and *DNMT3B5* (Hansen et al., 1999; Robertson et al., 1999). *DNMT3B* expression can be detected in several tissues but occurs at much higher levels in testis and thymus. The C-terminal region of the corresponding protein (MT3β) contains five highly conserved DNA methyltransferase motifs (I, IV, VI, IX, and X) (Xie et al., 1999) and a motif (VIII) with a much weaker similarity (Hansen et al., 1999). Functions for these motifs in the transmethylation reaction, such as cofactor binding, are inferred from crystallography data for other methyltransferases. The N-terminal region of MT3β contains a cysteine-rich domain that shows homology to the zinc-finger helicase region found in the ATRX protein (Picketts et al., 1996) that might mediate protein–protein or protein–DNA interactions. Closer to the N-terminus of MT3β is a PWWP domain that may also be involved in protein–protein interactions (Stec et al., 2000) but has recently been shown to bind DNA (Qiu et al., 2002).

More than 30 different ICF patients have been subjected to *DNMT3B* mutation analysis and at least 23 different mutations have been discovered (Fig. 49.2a) (reviewed by Hagleitner et al., 2008). ICF mutations in *DNMT3B* can be divided into three classes: (1) nonsense mutations that occur early in the transcript, (2) missense and splice-site mutations in the methyltransferase catalytic domain, and (3) a missense mutation near the PWWP domain. While the nonsense mutations are expected not to result in protein, the second class of mutations lead to proteins with compromised catalytic activity (Gowher and Jeltsch, 2002; Xu et al., 1999). A reported missense mutation near the PWWP domain in a Japanese ICF patient suggests an important role for this motif in MT3β function (Shirohzu et al., 2002). Studies in mice (Okano et al., 1999) have shown that complete MT3β deficiency is embryonic lethal; such lethality might also apply to humans. Consistent with the retention of some essential MT3β activity

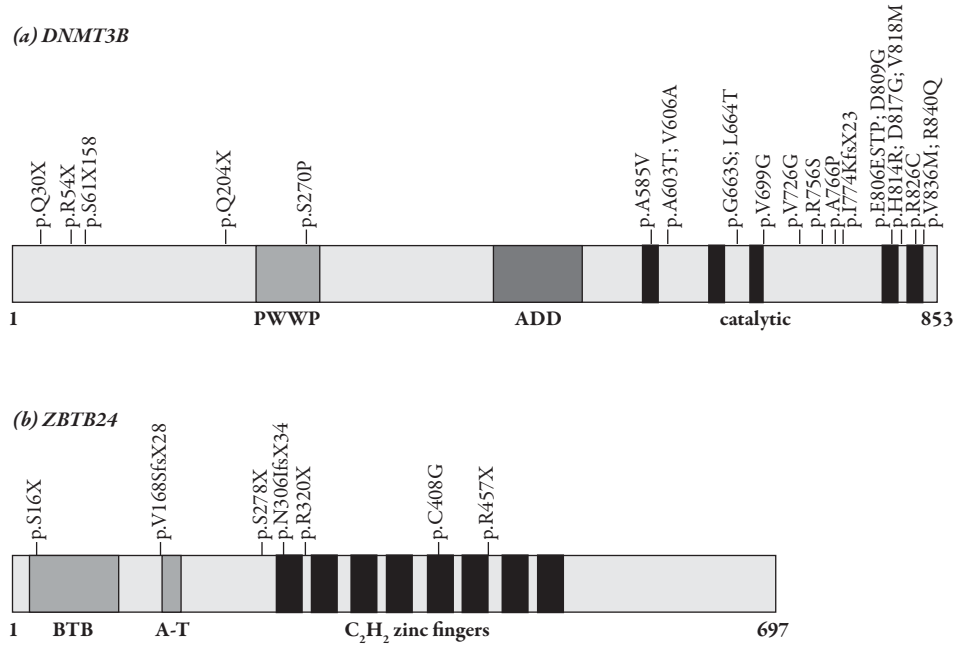

Figure 49.2 ICF mutations in *DNMT3B* (a) and *ZBTB24* (b). Codon numbers for mutations in *DNMT3B* refer to the 3B1 isoform (GenBank accession no. AF156488) (Hagleitner et al., 2008; Hansen et al., 1999). Gene structure and mutation data for *ZBTB24* are described in de Greef et al. (2011).

in ICF patients, all three predicted null mutations described so far are accompanied by a missense mutation in the other allele (Wijmenga et al., 2000; Xu et al., 1999). Bisulfite methylation studies of ICF cells also support residual methyltransferase activity for missense mutant enzymes (Hassan et al., 2001). Furthermore, some of the missense mutations have been shown to produce proteins with significant methyltransferase activity in vitro (Gowher & Jeltsch, 2002). Residual MT3β activity was also observed in mouse models in which specific point mutations observed in ICF patients were introduced in *Dnmt3b* (Ueda et al., 2006).

Based on the rarity of the disease and the finding of consanguinity in some ICF families, the early prediction was that most ICF cases would involve homozygous mutations derived from a limited number of founders. Only 8 of 17 ICF patients were found to have homozygous mutations, however, and all differed from each other; the majority of *DNMT3B* mutations, therefore, appear to have arisen from several unique events. Alternatively, it is possible that the number of gene carriers—and in turn the number of potential ICF patients—is much larger than expected; some mutations may be incompatible with life in certain heterozygous combinations, while others may lead to extremely mild phenotypes that have not been connected to ICF syndrome. Phenotype–genotype relationships have yet to be confidently uncovered because of the limited set of patients with known mutations, many of whom are compound heterozygotes. Additionally, phenotypic variability is likely for any given mutation because the corresponding molecular defects are predicted to derive from epigenetic abnormalities that are intrinsically variable.

DNMT3B FUNCTION

The primary defects leading to phenotypic consequences in ICF are predicted to result from abnormal escape from transcriptional silencing due to lack of methylation by MT3β. Hypomethylation and escape from silencing in ICF cells has been demonstrated for certain genes on the inactive X chromosome (Gartler & Hansen, 2002; Hansen et al., 2000; Tao et al., 2002), the Y chromosome (Hansen et al., 2000), an autosomal repetitive element (Kondo et al., 2000), and an autosomal cancer-testis gene (Tao et al., 2002). Hypomethylation in the promoter region is not sufficient for escape to occur in many cases because silencing is maintained unless accompanied by advanced replication (Hansen et al., 2000). Although it is not clear how these particular genes could be responsible for the ICF phenotype, it is likely that a similar mechanism is responsible for genes that do play key roles in facial development and the immune system.

Abnormal gene regulation could also result from hypomethylation at pericentromeric regions or other repetitive, CpG-rich regions by a mechanism similar to the phenomenon of position-effect variegation found in yeast, *Drosophila*, and mammalian systems (Kleinjan & van Heyningen, 1998; Wakimoto, 1998). Genes of this type would be affected in *cis* because they are within or near repetitive regions that are normally hypermethylated and late replicating. In addition to transcriptional regulation abnormalities, immunoglobulin deficiency in ICF could potentially result from a failure to achieve productive germline rearrangements critical to B- and T-cell development because of hypomethylation within the rearrangement loci (Engler et al., 1993; Mostoslavsky et al.,

1998; Whitehurst et al., 2000). This could lead to defective B-cell differentiation, lack of memory B cells, and impaired B-cell negative selection (Blanco-Betancourt et al., 2004).

In the immune system alone, the list of potential candidate genes for dysregulation with ICF-like consequences is quite long. One hope for finding key genes involved in ICF was the cDNA microarray approach for large-scale studies of gene expression in normal and ICF cells. Such data were expected to help identify key pathways affected in ICF that might be targeted for therapeutic intervention. Several attempts have been made to identify genes that are specifically deregulated in ICF syndrome (Ehrlich et al., 2001, 2008; Jin et al., 2008). Genes involved in diverse functions such as development, chromatin structure and transcriptional activity, signaling, immunity, and defense were identified as transcriptionally deregulated, and many of them could contribute to the primary phenotype of ICF syndrome. Interestingly, in only a few of the genes that were transcriptionally upregulated, subtle demethylation of their promoter regions could be detected. Promoters of some upregulated genes showed clear changes in histone modifications, including loss of repressive chromatin marks such as histone H3 lysine 27 trimethylation and the polycomb repressive complex PRC2 and gain of the active histone H3 lysine 9 acetylation marker (Jin et al., 2008).

Some of these transcriptionally deregulated genes show altered nuclear distribution in ICF LCLs. Some genes in the pseudoautosomal region 2 (PAR2) of human chromosomes X and Y showed an abnormal localization within the chromosome territory when differentially expressed (Matarazzo et al., 2007). In addition, a recent report also indicates that subtelomeric regions are hypomethylated in ICF syndrome, which is associated with abnormally short telomeres and enhanced expression of telomeric-repeat-containing RNA (Yehezkel et al. 2008). Collectively, these results indicate that defects in *DNMT3B* have a much wider genomic implication than previous anticipated from the first observations of pericentromeric defects.

MT3β appears to have several interacting partners (Geiman et al., 2004a, 2004b; Gopalakrishnan et al., 2009; Kim et al., 2002), including other DNA methyltransferases (MT1 and MT3α), histone deacetylases (HDAC1 and HDAC2), heterochromatin protein 1 alpha, components of a condensin complex (hCAP-C, hCAP-E, and hCAP-G), a chromatin remodeling protein (hSNF2H), a centromere protein (CENP-C), and a chromokinesin homolog (KIF4A). These interactions, however, are typically mapped to the N-terminal portion of the protein, whereas most ICF mutations occur in the C-terminal catalytic domains, indicating that the relevance of these interactions for ICF pathology needs further exploration. Interactions with the DNMT3L protein, however, do have clear relevance with respect to ICF mutations. DNMT3L is a DNMT3 family member that, although catalytically inactive itself, stimulates MT3β activity through interaction with the C-terminus. Two reported ICF mutations decrease the affinity between both proteins and strongly decrease the stimulation of MT3β by DNMT3L (Xie et al., 2006).

Also relevant to ICF mutations is the finding that MT3β is SUMOlyated and normally helps localize the protein to chromatin; the S270P ICF mutation in the N-terminal PWWP domain results in an abnormal diffuse nuclear localization and constitutive activation of nuclear factor kappa-B (Park et al., 2008).

ZBTB24 GENE STRUCTURE AND MUTATIONAL ANALYSIS

The *ZBTB24* gene contains 7 exons that span a region of about 22 kb on chromosome 6. It encodes a transcript of 5.5 kb. *ZBTB24* is ubiquitously expressed, but the highest levels of expression can be detected naïve B cells (de Greef et al., 2011). The ZBTB24 protein contains a BTB protein–protein interaction domain (bric-a-bric, tram-track, broad complex), a DNA-binding A-T hook domain, and eight C2H2 zinc-finger domains (Fig. 49.2a). BTB domains can be found at the N-terminus of other zinc-finger proteins, where they are involved in homomeric or heteromeric dimerization (Bardwell & Treisman, 1994) and transcriptional repression (Deweindt et al., 1995). A-T hook domains and C2H2 zinc-finger domains are DNA-binding motifs.

Seven of 11 ICF2 patients, mostly unrelated, were found to have mutations in *ZBTB24*. These mutations create premature stop codons in all but one patient. The remaining mutation replaces a conserved cysteine for a glycine in one of the conserved C2H2 zinc-finger domains of ZBTB24. This strongly suggests that loss-of-function mutations in *ZBTB24* underlie the ICF2 syndrome.

ZBTB24 FUNCTION

Although ZBTB24 has not been studied in detail, it is a member of a family of more than 40 human ZBTB proteins, some of which have been shown to be involved in hematological differentiation (Stogios et al., 2005). Perhaps the most studied ZBTB protein in the context of hematological differentiation is BCL6 (ZBTB27). BCL6 was identified as being deregulated in certain B-cell lymphomas through chromosomal translocations (Ye et al., 1995) and is required for germinal center formation (Dent et al., 1997). Other ZBTB proteins that have been mechanistically linked to hematological development and malignancy are BCL6B (ZBTB28), PATZ1 (ZBTB19), ZBTB7B, and ZBTB32 (Bilic et al., 2006; He et al., 2005; Kang et al., 2005; Manders et al., 2005; Piazza et al., 2004; Sun et al., 2005; Takamori et al., 2004). Future functional studies of ZBTB24 are, therefore, likely to further our understanding of the pathophysiology of ICF syndrome.

ANIMAL MODEL

Additional evidence that MT3α and MT3β do, indeed, have de novo methyltransferase activity came from studies in mice, in which both genes were inactivated by homologous recombination (Okano et al., 1999). The expression levels of *Dnmt3a* and *Dnmt3b* are extremely high in undifferentiated embryonic stem cells but are rapidly downregulated after differentiation such that much lower levels are found in adult somatic tissues. Both *Dnmt3a* and *Dnmt3b* appear to be essential for

mouse development, as the *Dnmt*3b$^{-/-}$ mice die before post-conception day 11.5 and the *Dnmt3a*$^{-/-}$ mice die perinatally. The *Dnmt3a* and *Dnmt3b* genes exhibited some overlapping methylation target sites, but *Dnmt3b* deficiency specifically decreased methylation at pericentromeric minor satellite repeats, similar to the hypomethylation seen in ICF syndrome patients at analogous satellite repeats.

More recently, ICF knock-in mice were generated by introducing two ICF mutations into the murine *Dnmt3b* gene by homologous recombination and the catalytic domain of MT3β was also deleted as part of the study (Ueda et al., 2006). While the deletion model resulted in embryonic lethality similar to the *Dnmt3b* knockout alleles, mice homozygous for the ICF mutations developed to term and had phenotypes comparable with ICF syndrome, including hypomethylation of repetitive DNA, craniofacial anomalies, and T-cell death by apoptosis. Remarkable differences were also noted: while ICF patients typically show severely reduced immunoglobulin counts, these mice show mainly defects in the T-cell lineage (Ueda et al., 2006). In ICF syndrome, T-cell defects are rarely described. Nevertheless, these mice will likely prove useful for further studies of the pathogenic mechanisms underlying this disorder; presumably, mouse models of ZBTB24 deficiency will also contribute to our understanding.

CONCLUDING REMARKS

On the basis of our current knowledge, it is likely that a compromised MT3β methyltransferase leads to hypomethylation of specific chromosomal loci, deheterochromatinization, advanced replication, and transcriptional activation of key genes whose dysregulation affects facial development and the immune system. A compromised ZBTB24 protein presumably affects overlapping genes and/or pathways to bring about the phenotypes shared by ICF1 and ICF2 patients. Discovery of such genes should further our understanding of these phenotypes and of the normal role of these proteins in methylation-dependent transcriptional regulation. Examination of the molecular properties of these proteins and their interacting partners will help explain the apparent specificity for hypomethylation defects and provide candidate genes that might be mutated in ICF patients who were not found to have mutations in either of the two known genes. Although the syndrome appears to be quite rare, increased ascertainment may develop as awareness of ICF increases and diagnosis of immunodeficiency includes more cytogenetic and methylation studies. The ICF syndrome provides one of the first examples of a human disease whose major defect alters the epigenetic structure and regulation of the genome, thus providing us with excellent model systems for study. Because proper epigenetic regulation is crucial for development and differentiation, explication of the epigenetic alterations in ICF and their functional consequences should have implications for a large spectrum of human disease.

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50.

INTRODUCTION TO GRANULOCYTE DISORDERS

Karl Welte, Cornelia Zeidler, and David C. Dale

Neutrophils play a major role in orchestrating the inflammatory response, in the resolution of inflammation, and in wound healing, in addition to simply killing microorganisms (Borregaard et al., 2007; Dale et al., 2008). The bone marrow of healthy individuals produces 1 million neutrophils/kg/minute. This is a highly regulated and dynamic process that requires specific hematopoietic growth factors and a functional bone marrow microenvironment. Hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3) stimulate receptor-dependent intracellular signaling pathways that lead to activation of lineage-specific transcription factors (Tenen et al., 1997). In steady state, differentiation depends on the transcription factors LEF-1 and C/EBP alpha (Skokowa et al., 2006), whereas C/EBP epsilon is required for terminal differentiation and expression of secondary granule proteins (Gombart et al., 2005). With stress or infections, C/EBP beta is also required (Hirai et al., 2006).

The entry and exit of neutrophil into and from the bloodstream is also a highly regulated process. Receptor ligand pairs such as VLA-4 binding to VCAM-1 or fibronectin and SDF-1/CXCL 12 (Eash et al., 2009) binding to CXCR-4 retain neutrophils and their progenitors in the marrow. Several chemokines regulate the movement of these cells from the marrow to the blood.

Once released from the marrow, the neutrophil half-life in the blood is only 6 to 10 hours. The cells in the vascular compartment are approximately equally distributed between the circulating and marginal pools. Neutrophils exit from the marginal pool and enter the tissues by first attaching to the vascular endothelial cells and then squeezing out between them. Integrins and selectins expressed on the surface of neutrophils direct these functions. In the tissues neutrophils ingest and kill invading microorganisms through the generation of H_2O_2 and oxygen radicals and the actions of a variety of enzymes stored in the neutrophils' primary and secondary granules. Immunoglobulins and complement proteins facilitate the process of ingestion and killing, which depends primarily on an intact system for generating oxygen radicals. Neutrophils also secrete cytokines and chemokines, such as IL-1, IL-8, TNF, VEGF, and CXCL2, that support resolution of inflammation and healing of damaged tissue (Borregaard et al., 2007; Dale et al., 2008).

This chapter focuses on recognizing neutrophil disorders, both of numbers and functions. Subsequent chapters (51–53) deal in greater detail with the clinical features, pathophysiology, and treatment of these conditions.

NEUTROPENIA

Normally the circulation blood neutrophil count is between 1,800 and 7,000/uL both in children and adults. Neutropenia (neutrophil count <1,800/uL) is easily detected by a routine complete blood count. Severe neutropenia is defined as an absolute decrease in the number of circulating neutrophils in blood to levels below 500/uL (Welte & Boxer, 1997). In most disorders affecting neutrophil numbers, neutropenia is chronic and often severe. The hallmark of severe chronic neutropenia is increased susceptibility to bacterial infections of the oropharynx (particularly the periodontal tissues), the respiratory tract, and the skin. The most common manifestations are mouth ulcers, gingivitis, sinusitis, pharyngitis, cellulitis, and cutaneous abscesses. When neutropenia is very severe and the patient cannot generate neutrophils in response to the stress of infection, abscesses of the lung and liver, other deep tissue infections, and bacteremia may occur.

Neutropenic colitis with invasion by anaerobic pathogens such as *Clostridium* species is a severe and often fatal complication. Bacteremia is otherwise uncommon, unless there is concomitant monocytopenia or other defects in host defenses. The most commonly isolated bacteria from neutropenic patients are *Staphylococcus aureus* and gram-negative bacteria. Fungal infections usually follow periods of antibiotic treatment.

CONGENITAL NEUTROPENIA

Congenital neutropenia (CN) comprises a heterogeneous group of bone marrow failure syndromes characterized by a maturation arrest of myelopoiesis at the level of the promyelocyte/myelocyte stage, with peripheral blood absolute neutrophil counts (ANC) below 0.5×10^9/L. CN is usually recognized when a complete blood count with differential is performed in an infant or young child with recurrent fever and signs and symptoms of recurrent infections (Dale et al., 1993; Welte & Dale, 1996; Welte et al., 2006). In this setting, the differential diagnosis includes alloimmune, autoimmune, or drug-induced neutropenia and viral infections. The most useful assessments are a careful medical history and thorough examination of the patient, review of previous blood counts, if available, and a bone marrow examination. As noted above, in an infant or young child with several days or weeks of severe chronic neutropenia, the finding of "maturation arrest" in the marrow is highly suggestive of the diagnosis of congenital neutropenia (see Chapter 51).

Recent research has permitted a genetic approach to classifying CN (Table 50.1) and has also shed light on the molecular mechanisms controlling the development and function of neutrophils. From a clinical point of view, two major groups of inherited neutropenia disorders can be distinguished: CN without major additional organ system manifestations, and CN occurring as part of multisystem disease. CN is also classified by the pattern of inheritance. For example, autosomal dominant CN is attributable to neutrophil elastase gene (*ELA2*) mutations and represents approximately 50 to 60 percent of all cases of CN (Dale et al., 2000). Because the *ELA2* gene is expressed solely in myeloid tissues, neutrophils are the only tissue affected with mutations of this gene.

Autosomal recessive CN is less common, representing up to 30 percent of patients with CN, depending on the ethnic makeup of the population. The genes associated with autosomal recessive severe CN—for example, *HAX1* (Klein et al., 2007) and *G6PC3* (Boztug et al., 2009)—are widely expressed, and patients with these mutations may have other congenital abnormalities. From a pathophysiological perspective, ineffective production of neutrophils due to cell loss or apoptosis during development is a common feature of these diseases (Dale et al., 2009). Table 50.1 lists genetic mutations associated with CN.

Both autosomal dominant and autosomal recessive CN are now considered as a preleukemic condition, with a cumulative incidence for leukemia estimated as high as 25 percent after 20 years of observation (Rosenberg et al., 2006). The individual risk for each genetic subtype and the genotype–phenotype associations are not yet clear because the number of patients tested for an underlying genetic defect is still limited. Both groups of patients, HAX1-CN and ELA2-CN, develop leukemia in a similar frequency. Acquired G-CSF receptor (*CSF3R*) mutations are detected in approximately 80 percent of CN patients who developed acute myeloid leukemia, suggesting that these mutations are involved in leukemogenesis (Ancliff et al., 2003; Dong et al., 1995; Germeshausen et al., 2007).

CYCLIC NEUTROPENIA

Cyclic neutropenia is a unique form of CN. It is an autosomal dominant disorder characterized by regular oscillations of blood neutrophil counts (between 0 and 2,000/uL), with a usual period length of 21 days. Fever, mouth ulcers, and infections, sometime fatal, are common during the neutropenic periods. More than 80 percent of well-documented cases of cyclic neutropenia are attributable to mutations in the neutrophil elastase (*ELA2*) gene (Horwitz et al., 2007) (Chapter 51).

CONGENITAL NEUTROPENIA ASSOCIATED WITH SYNDROMAL FEATURES

A number of immune deficiency syndromes are associated with neutropenia. Usually, the neutropenia is a secondary and variable feature of the syndrome. In brief, some key features for recognition of these conditions are as follows.

NEUTROPENIA ASSOCIATED WITH LYMPHOID CELL DYSFUNCTION

Reticular dysgenesis is caused by mutations in the adenylate kinase (*AK2*) gene and is characterized by early differentiation arrest in the myeloid lineage associated with severe combined immunodeficiency and sensorineural hearing loss. It is characterized by neutropenia and lymphopenia and defective humoral immunity (Lagresle-Peyrou et al., 2009; Pannicke et al., 2009) (Chapter 18).

Wiskott-Aldrich syndrome is caused by mutations in the *WAS* gene (Chapter 43). In rare cases, neutropenia is associated with activating mutations in the WAS protein and may be the presenting or sole manifestation (Ancliff et al., 2006; Devriendt et al., 2001).

Warts, Hypogammaglobulinemia, Immune deficiency, Myelokathexis (WHIM) syndrome is defined as dysgranulopoiesis associated with hypogammaglobulinemia and dermal papillomavirus infections (warts) (Gorlin et al., 2000). WHIM is caused by mutations in the chemokine receptor gene *CXCR4*, the receptor for SDF-1 (Hernandez et al., 2003). The neutrophils show vacuoles, prominent granules, and nuclear hypersegmentation with pyknotic-appearing nuclear lobes (Chapter 40).

X-linked hyper-IgM syndrome is caused by mutations in the *CD40L* gene that, in addition to neutropenia, cause defects in switching from IgM to IgG, IgA, and IgE (Etzioni et al., 2004) (Chapter 26).

Table 50.1 CAUSES FOR CHRONIC NEUTROPENIA

	GENETIC DEFECT	RECESSIVE (R-CN)	DOMINANT (D-CN)	NEUTROPENIA PLUS
ELA2 CN	*ELA2*	-	+	Preleukemic syndrome
HAX1 CN	*HAX1*	+	-	Preleukemic syndrome
Neutropenia with *WASP* mutation	*WASP*	X-linked	-	Monocytopenia, platelets normal
Neutropenia associated with metabolic syndrome				
Shwachman-Diamond syndrome	*SBDS*	+	-	Exocrine pancreas insufficiency, short stature, bone anomalies, anemia, thrombocytopenia, leukemic transformation
Barth syndrome	*Taz 1*	X-linked	-	Dilated cardiomyopathy, skeletal myopathy, short stature, 3-Methylglutaconic aciduria
GSD 1b*	Glucose-6-phosphate-translocase	+	-	Hypoglycemia, lactic acidosis
G6PC3 CN	*G6PC3*	+	-	Short stature, cardiac or urogenital anomalies, visibility of subcutaneous veins
Neutropenia with lymphoid cell dysfunction				
Congenital neutropenia with *GFI-1* mutation	*GFI-1*	-	+	B-/T-cell deficiency
WHIM syndrome	*CXCR4*	-	+	Myelokathexis, IgG deficiency, warts
Hyper-IgM syndrome	*CD40-L*	X-linked	-	IgG, IgA, IgE deficiency
Reticular dysgenesis	*AK2*	+	-	Severe combined immunodeficiency, sensorineural hearing loss
Neutropenia with pigmentation defects				
Griscelli syndrome	*Rab27a*	+	-	Partial albinism, IgG deficiency despite normal lymphocyte numbers, hemophagocytosis
Chediak-Higashi syndrome	*CHS* gene	+	-	Albinism, T/NK + chemotaxis defect
Hermansky-Pudlack syndrome	*AP3B1*	+	-	Partial albinism, short stature, IgG deficiency, hemorrhagic diathesis
Hermansky-Pudlack-like syndrome	*p14*	+	-	Partial albinism, short stature, IgG deficiency

CN= chronic neutropenia; GSD 1b = glycogen storage disease type 1b

NEUTROPENIA ASSOCIATED WITH PIGMENTATION DEFECTS

Chediak-Higashi syndrome (CHS) (Chediak et al., 1952; Higashi et al., 1954) and Griscelli syndrome (Menasche et al., 2000) have variable degrees of neutropenia and susceptibility to infections. CHS is characterized by oculocutaneous albinism, giant lysosomes in neutrophils, and neuropathy. Hermansky-Pudlak syndrome type 2 (HPS2) is also characterized by decreased numbers of NK cells, and p14 deficiency is characterized by persistently reduced peripheral blood neutrophil counts. In contrast to CN with myeloid maturation arrest, CHS, HPS2, and p14 deficiency show presence of neutrophils in the bone marrow. CHS is caused by mutations in the *CHS* gene; HPS2 by mutations in the *AP3B1* gene (Jung et al., 2006), which encodes an adapter protein involved in shuttling granule proteins; and p14 deficiency by a mutation in the *p14* gene (Bohn et al., 2007), also encoding for an endosomal adaptor protein (Chapter 51).

NEUTROPENIA ASSOCIATED WITH METABOLIC ABNORMALITIES

Glycogen storage disease type Ib (GSD1) is caused by mutations in the glucose-phosphate-translocase gene. GSD1 patients show typical features of glycogen storage disease (hypoglycemia, lactic acidosis, hepatomegaly) (Melis et al., 2005). In contrast, patients with *G6PC3* deficiency caused by mutations in the *glucose-6-phosphatase subunit 3* gene (Boztug et al., 2009) do not develop symptoms of glycogen storage. However, they show increased visibility of superficial veins and developmental defects of the cardiovascular and urogenital systems. The pathophysiological mechanism of neutropenia may be due to endoplasmic reticulum stress and increased apoptosis of myeloid cells (Chapter 51).

EXOCRINE PANCREAS INSUFFICIENCY (SHWACHMAN-DIAMOND SYNDROME)

Patients with Shwachman-Diamond syndrome (SDS) suffer from exocrine pancreas insufficiency, short stature,

chondrodysplasia, and severe neutropenia (Shimamura et al., 2006; Shwachman et al., 1964). SDS is caused by mutations in the *SBDS* gene (Boocock et al., 2003), which leads to defective ribosomal maturation and defective protein synthesis. Patients with SDS are at high risk for developing leukemia (Dror et al., 2002).

DISORDERS OF NEUTROPHIL FUNCTION

Inherited disorders of neutrophil function are attributable to mutations in the many specific components of the neutrophil that are responsible for the formation of granules and granule proteins, the internal and cell surface proteins and energy components necessary for neutrophil trafficking, and the enzymes essential for the metabolic burst and generation of oxygen radical and hypochlorous acid for microbial killing (Chapter 52). Some disorders affect both neutrophil production and function.

Identification of specific defects in neutrophil function is complex. It begins with a complete history and physical examination because many common conditions, such as diabetes, renal and vascular diseases, and conditions causing chronic edema, predispose patients to infections by affecting neutrophil trafficking and function. A history of bacteremia and the specific organisms isolated are useful. Bacteremia with an encapsulated organism is a clue that the patient may have a phagocytic defect, most commonly a defect in immunoglobulins or complement proteins, or in the clearance function of the spleen. It is very important to obtain complete blood counts when the patient is in good health and during illness. Some patients with functional defects, for example CHS, are neutropenic and fail to develop neutrophilia in the face of severe infection. Others, for example patients with leukocyte adhesion deficiency syndrome, have extreme neutrophilia with their recurrent infections. It is critical to perform a careful examination of the blood cells by light microscopy, looking at the presence and size of the granules. Tests of the metabolic burst associated with phagocytosis, as well as screening tests for chronic granulomatous disease, are now widely available, but further cellular and biochemical testing generally requires referral to a research laboratory. Genetic analysis is best performed after consultation with a specialist.

DEFECTS IN FORMATION OF GRANULES AND GRANULE PROTEINS

There are a number of defects in the formation of neutrophil granule and granule proteins. CHS is an autosomal recessive disorder affecting many organ systems, identified readily by the giant granules in neutrophils. In the specific granule deficiency syndrome, an autosomal recessive disorder, the secondary granules and all of their constituents are missing, i.e., lactoferrin, alkaline phosphatase and defensins. The defect can be seen with light microscopy on Wright-stained blood smears. In myeloperoxidase deficiency, standard histochemical staining readily reveals the abnormality.

DEFECTS IN NEUTROPHIL TRAFFICKING

Leukocyte adhesion deficiency syndrome (LAD) (Chapter 53) should be suspected in a child with recurrent high fevers, leukocytosis and neutrophilia, and recurrent soft tissue infections (Arnaout, 1990; Crowley et al., 1980). Characteristically there is evidence of soft tissue inflammation but delay in the formation of pus. LAD I is an autosomal recessive disorder in which neutrophil adhesion, chemotaxis, and ingestion of opsonized microbes are impaired because of mutations in the gene for CD18, ß subunit of the ß2 integrins (Dana et al., 1984). Diagnosis is established by flow cytometry to detect expression of CD18. LAD II is a clinically similar syndrome caused by defective selectin-mediated adhesion due to deficient sialyl-Lewis X ligand, and LADIII is caused by mutations in *Kindlin 3*, which is crucial for β2 integrin adhesiveness (Chapter 53).

In WHIM syndrome, mutations in *CXCR4* result in abnormal trafficking of neutrophils and lymphocytes, resulting in severe neutropenia and lymphopenia, with predominantly apoptotic neutrophils seen in the circulation (Chapter 40).

DEFECTS IN THE METABOLIC BURST AND MICROBIAL KILLING

Patients with defects in the metabolic burst and microbicidal mechanisms usually present with recurrent fevers and pulmonary, gastrointestinal, and cutaneous infections. The hallmark of these disorders is the development of chronic inflammatory granulomas widely distributed in the body. The identity of the organisms causing the infections is helpful in making this diagnosis. For example, in chronic granulomatous disease, infections by fungi, such as *Aspergillus* species as well as *S. aureus* and other catalase-positive microorganisms, are common (Chapter 52).

Several molecular and genetic defects cause a similar syndrome: defects in the components of NADPH-oxidase, G6PD, MPO, glutathione reductase, and glutathione synthetase. Chronic granulomatous disease is an inherited disorder of neutrophil function in which generation of superoxide by the respiratory burst oxidase is absent or markedly deficient (Malech et al., 2007). Definition of the specific defect is important for the prognosis and care of these patients (Chapter 52).

EVALUATION OF PATIENTS WITH DISORDERS OF GRANULOPOIESIS

Many of the phagocyte disorders have similar clinical manifestations, which can even overlap with disorders of lymphocyte function. Patients with an unusual frequency of bacterial infections or with infections with exceptional severity should be evaluated for disorders of granulopoiesis. The first test to consider is a peripheral blood neutrophil count. If the number is sufficient or even elevated, phagocyte function assays should be performed, such as the nitroblue-tetrazolium test, or flow-based assays for superoxide generation or the expression of CD18 surface glycoprotein.

If neutrophil counts are below 500/uL, a neutropenia workup is indicated, starting with a search for antineutrophil

antibodies. If there are no antibodies detectable, bone marrow aspiration should be performed to search for maturation arrest, typical for congenital neutropenias. In addition, genetic testing for mutations in the genes for *ELA2*, *HAX1*, and others should be performed. Cyclic neutropenia is diagnosed by the cyclic pattern of serial blood neutrophil counts. In neutropenias associated with specific syndromes, skeletal anomalies, or skin anomalies (e.g., pigmentation defects), workup for heart or metabolic anomalies should be performed.

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51.

SEVERE CONGENITAL NEUTROPENIA

Christoph Klein

CLINICAL FINDINGS

In 1950, the Swedish pediatrician Rolf Kostmann described eight children suffering from lethal bacterial infections due to almost complete absence of mature neutrophil granulocytes (Kostmann, 1950, 1956). The disease was transmitted as an autosomal recessive trait and was characterized by a block in myeloid cell maturation at the stage of promyelocytes (Fig. 51.1a). Historically, this report represents the first description of a human primary immunodeficiency disorder. Affected patients are prone to severe and recurrent bacterial infections such as otitis media, bronchitis, pneumonia, osteomyelitis, or cellulitis (Fig. 51.1b). During long periods of persistent neutropenia (i.e., in untreated conditions), patients are at risk for fungal infections (Al-Kindi et al., 2008; Alvarez-Moya et al., 2009). A characteristic feature of infected sites is the absence of pus. Many patients suffer from chronic gingivitis and tooth decay. In addition, decreased bone mineral density, leading to osteopenia or osteoporosis and an increased propensity to fractures, is a common clinical problem (Borzutzky et al., 2006; Yakisan et al., 1997).

Congenital neutropenia may be an isolated finding or may be seen in association with other syndromal features, such as hypopigmentation, neurocognitive delay, pancreatic insufficiency, cardiac or urogenital malformations, or deficiency of adaptive immunity (Table 51.1). Therefore, a comprehensive clinical examination is mandatory. The identification of genetic defects has started to illuminate the pathophysiology of severe congenital neutropenia (SCN) syndromes and specific risk factors for long-term complications (Klein, 2011). Recent insights into the function of SCN genes have also been made possible by in vivo studies in animal models.

MOLECULAR GENETICS

NEUTROPENIA WITHOUT SYNDROMAL FEATURES

SCN and Mutations in ELA2/ELANE

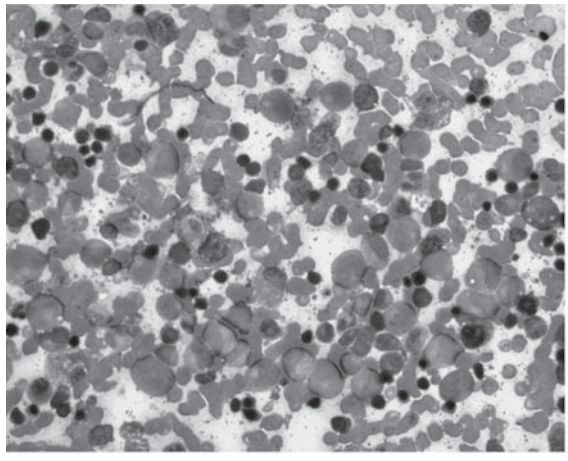
The team of Marshall Horwitz and David Dale was the first to identify a molecular defect in patients with cyclic neutropenia (Horwitz et al., 1999) and SCN (Dale et al., 2000). Using a genome-wide linkage analysis approach in 13 families with cyclic neutropenia, these investigators identified heterozygous mutations in the gene encoding neutrophil elastase (ELANE—ELA2-neutrophil expressed). ELANE is a serine protease exclusively expressed in myeloid cells and is found as a constitutive protein in azurophilic granules in neutrophil granulocytes. Upon stimulation of granulocytes, ELANE is released and performs a variety of functions. By cleaving bacterial proteins, ELANE serves as a defense mechanism. In addition, ELANE may bind to and cleave host proteins such as N2N, a member of the Notch family of proteins (Duan et al., 2004), G-CSF and G-CSF receptor (Hunter et al., 2003), coagulation factors, and extracellular matrix proteins (Faurschou & Borregaard, 2003). ELANE also displays enzymatic activity inside the cells. Processing of PML-RAR alpha may directly affect the differentiation control of myeloid cells (Lane & Ley, 2003).

So far, more than 50 mutations in *ELANE* have been identified in patients with SCN or cyclic neutropenia (Fig. 51.2) (Horwitz et al., 2007). Mutated ELANE in patients with SCN acts as a dominant-negative factor. *ELANE* mutations can be acquired or transmitted in an autosomal dominant mode(Boxer et al., 2006). A number of families with somatic mosaicism have also been reported (Ancliff et al., 2002; Germeshausen et al., 2001b).

Table 51.1 ORGAN INVOLVEMENT IN CONGENITAL NEUTROPENIA SYNDROMES

CN VARIANT	CONGENITAL NEUTROPENIA	OSTEOPENIA	SKELETAL SYSTEM (GROWTH DELAY/ DYSMORPHIC FEATURESBV	SKIN/ HAIR	NEUROLOGICAL SYSTEM	CARDIOVASCULAR SYSTEM	UROGENITAL SYSTEM	GASTROINTESTINAL SYSTEM	ENDOCRINE SYSTEM	ADAPTIVE IMMUNE SYSTEM	MUTATED GENE
SCN-ELANE	•	•									ELANE
SCN-GFI1	•	•								•	GFI1
SCN-WAS	•									•	WAS
SCN-HAX1	•	•			•						HAX1
SCN-AK2	•				•					•	AK2
Glycogenosis Ib	•	•	•			•		•	•		SLC37A4
G6PC3 deficiency	•		•	•		•	•	•			G6PC3
Barth syndrome	•					•					TAZ
SBDS	•	•	•		•	•		•			SBDS
CHH	•	•	•	•	•			•		•	RBDS
CHS	•		•	•						•	LYST
GS type II	•			•						•	RAB27A
HPS II	•		•	•						•	AP3B1
P14-deficiency	•		•	•						•	ROBLD3
Cohen syndrome	•		•	•							COH1
Poikiloderma with neutropenia	•		•	•							C16orf57
Neutropenia-CMT-II	•				•						DNM2

Myeloid cells with mutated ELANE are prone to premature apoptosis (Aprikyan et al., 2003; Grenda et al., 2007; Massullo et al., 2005), yet the mechanisms of increased cell death remain incompletely resolved. Three nonmutually exclusive hypotheses have been raised: (1) disruption of proteolytic activity of ELANE, (2) mislocalization of the mutated protein, and (3) induction of the "unfolded protein response" due to misfolding of the mutated protein (Horwitz et al., 2007; Xia & Link, 2008). The "unfolded protein response" has evolved to protect cells against damaging effects of improperly folded proteins.



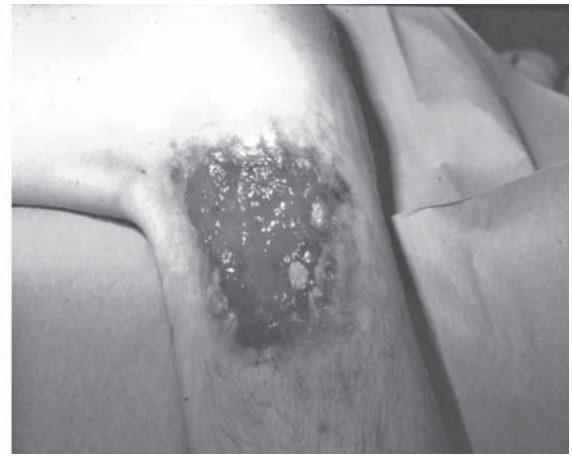


Figure 51.1 (a) Bone marrow smear showing myeloid maturation arrest. (Copyright from Nature Genetics.) (b) Necrotizing fasciitis. (Courtesy of K. Welte.)

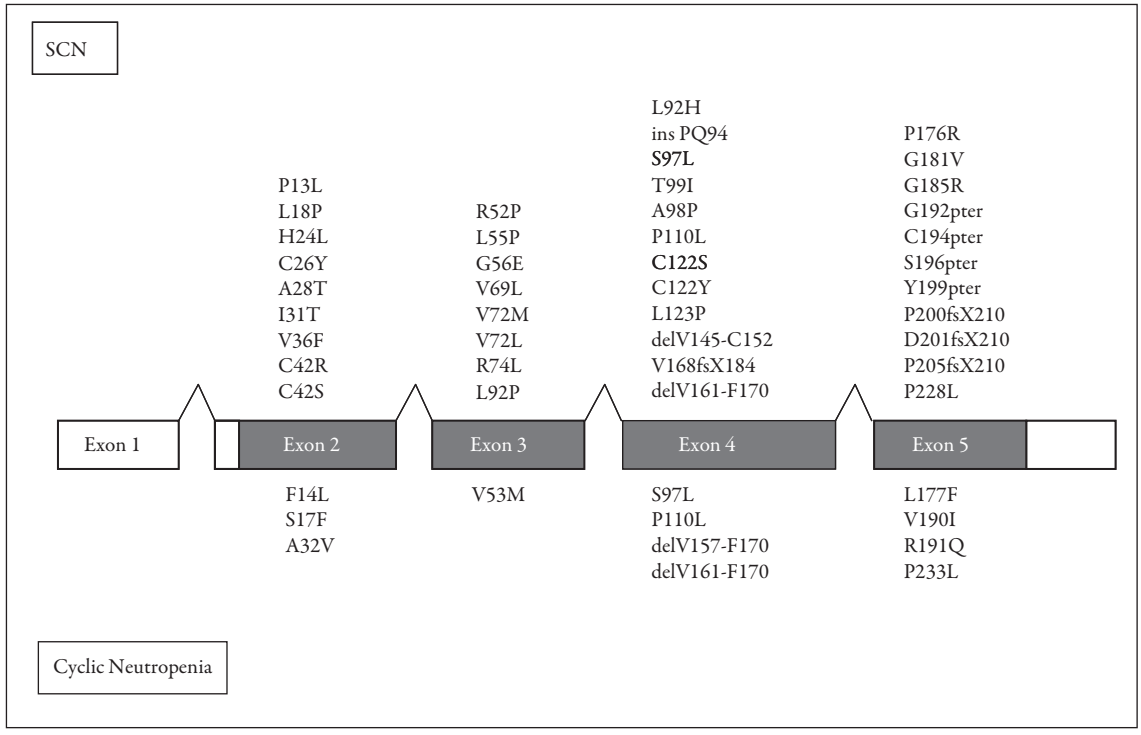


Figure 51.2 Synopsis of known mutations in ELANE.

Nascent proteins destined for secretory vesicles are directed to the endoplasmic reticulum (ER), where protein folding takes place (Ron & Walter, 2007). The unfolded protein response signal cascade is initiated by three ER-localized protein sensors: IRE1 alpha (inositol-requiring 1alpha), PERK (double-stranded RNA-dependent protein kinase [PKR])

-like ER kinase, and activating transcription factor 6 (ATF6). In case of ER stress, these sensors are activated and trigger a complex series of events destined to maintain the homeostasis of the ER and to promote protein folding, maturation, secretion, and ER-associated protein degradation. If these rescue mechanisms fail, the unfolded protein response will initiate apoptosis to protect cells and the organism from dysfunctional or toxic proteins (Zhang & Kaufman, 2008). Both myeloid cell lines engineered to express mutant ELA2 proteins and primary human cells from SCN-ELANE^{mut} patients showed increased biochemical signs of ER stress such as upregulation of Bip and cleavage of XBP-1 (Grenda et al., 2007; Kollner et al., 2006), strongly suggesting that the unfolded protein response is at least partially involved in the pathophysiological mechanisms explaining increased apoptosis in myeloid progenitor cells. Consistent with this hypothesis, neutrophils from patients expressing SCN-ELANE^{mut} show decreased transcription and translation of multiple granule-associated proteins, such as myeloperoxidase, lactoferrin, cathepsin G, and human neutrophil peptide (Donini et al., 2007), the bactericidal protein LL37 (Putsep et al., 2002), and neutrophil elastase itself (Skokowa et al., 2009a).

Despite these insights, many questions remain. For example, why do ELANE^{mut} alleles cause cyclic neutropenia in some patients and SCN in others? Why do some patients respond to G-CSF whereas others are considered nonresponders? Why do ELANE^{mut} alleles predispose to leukemogenesis? These clinically relevant questions must be addressed by future studies.

SCN and Mutations in HAX1

In his seminal paper, Rolf Kostmann noted that SCN was transmitted in an autosomal recessive mode of inheritance (Kostmann, 1950, 1956)—clearly distinct from the autosomal dominant inheritance pattern in ELANE^{mut} patients. This clinical observation was the first clue that SCN must comprise heterogeneous genetic defects. To investigate the molecular etiology of autosomal recessive SCN, we initiated a genome-wide linkage analysis in three consanguineous Turkish families and discovered homozygous mutations in HAX1 (HCLS1-associated protein X-1). Subsequently, biallelic mutations in HAX1 were identified in many SCN patients of various ethnic backgrounds, including the historic Swedish patients described by Kostmann (Fig. 51.3).

HAX1, a ubiquitously expressed antiapoptotic protein, was originally identified as a binding partner of HS1, a protein kinase involved in BCR-mediated signaling (Suzuki et al., 1997). In light of its distant sequence homology to members of the BCL2 family of antiapoptotic molecules and its predominant mitochondrial localization, the hypothesis has been raised that HAX1 might display an antiapoptotic function. In fact, a number of functional studies have confirmed a role for HAX1 in the maintenance of cellular viability (Sharp et al., 2002; Yedavalli et al., 2005). HAX1 regulates the inner mitochondrial membrane potential ($\Delta\Psi_m$), which is maintained by

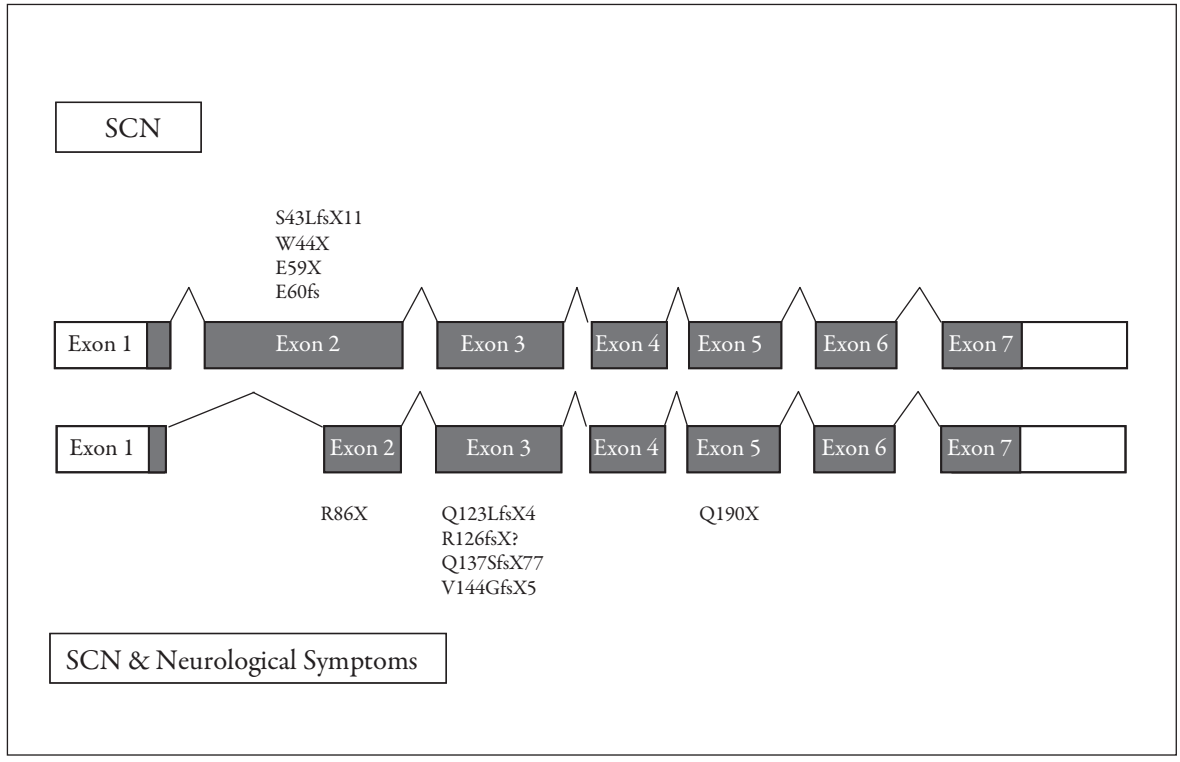


Figure 51.3 Synopsis of known mutations in HAX1.

an active ion gradient. HAX1-deficient cells display a lower threshold to dissipate their inner mitochondrial membrane potential, defining a point of no return for the apoptotic pathway. Loss of $\Delta\Psi_m$ triggers activation of Bax, release of cytochrome c, and activation of death-inducing caspases. In addition to its role in controlling the mitochondrial membrane potential, HAX1 interacts with a great variety of cytosolic proteins, such as cortactin and the polycystic kidney disease protein PKD2 (Gallagher et al., 2000), G alpha13 (Radhika et al., 2004), IL1 alpha (Kawaguchi et al., 2006), and integrin alphavbeta6 (Ramsay et al., 2007), suggesting that HAX1 may also coordinate signaling pathways in the cytosol. A number of viral proteins has also been shown to interact with HAX1—findings that have supported the hypothesis that viruses may have hijacked some of the endogenous functions of HAX1 to escape apoptosis (reviewed in Klein, 2011).

Important insights into the biology of HAX1 came from a murine model system (Chao et al., 2008). Murine hax1 controls parl-dependent conformational changes in htra2/omi, suggesting that hax1 plays a role in presenting htra2 to a proteolytic processing machinery. In contrast to human patients, hax1-deficient mice die shortly after reaching adolescence of neuronal degeneration. This observation has triggered a series of neurological investigations in human HAX1-deficient patients. Of note, Swedish patients originating from the families described by Kostmann suffer from cognitive disorders (Carlsson & Fasth, 2001). Neurological impairment, from mild developmental delay to severe epilepsy and devastating neurodegeneration, has been described in several SCN patients (Ishikawa et al., 2008; Matsubara et al., 2007; Rezaei et al., 2007). Further studies revealed a surprising genotype–phenotype correlation (Germeshausen et al., 2008). Two human HAX1 splice variants are known, giving rise to isoform A and isoform B. Isoform B is characterized by a splice event removing part of exon 2 and is preferentially expressed in neuronal cells. Mutations affecting only isoform 1 (i.e., mutations in exon 2) cause a phenotype restricted to congenital neutropenia, while mutations affecting both isoform 1 and isoform 2 lead to SCN associated with variable degrees of neurological impairment (Carlsson et al., 2008; Germeshausen et al., 2008).

Congenital Neutropenia and Mutations in GFI1

GFI1 (growth factor independent 1) is a zinc-finger transcription factor controlling differentiation of multiple hematopoietic and nonhematopoietic cells. Originally identified as a transcriptional repressor in T cells (Zweidler-Mckay et al., 1996), Gfi1 has become a key regulator of hematopoietic stem cell differentiation, as evidenced by defective myelopoiesis (Hock et al., 2003; Karsunky et al., 2002) and reduced stem cell self-renewal (Hock & Orkin, 2005) in Gfi1-knockout mice. In addition, Gfi1 is necessary to control intestinal endocrine cell differentiation and viability of neuronal cells (Shroyer et al., 2005; Tsuda et al., 2005).

Person et al. described two patients with heterozygous mutations in GFI1 (GFI1N382S), affecting the DNA-interacting domain of GFI1 (Person et al., 2003). Affected patients showed a myeloid maturation arrest, a paucity of mature neutrophils, and a propensity to develop recurrent infections. Recently, a murine model of the $GFI1^{N382S}$ mutant has been studied in greater detail, confirming a dominant-negative

block in granulopoiesis associated with dysregulation of CSF1 and its receptor (Zarebski et al., 2008).

Acting as a transcriptional repressor, GFI1 controls multiple target genes and regulatory micro-RNAs (Hock et al., 2003; Hock & Orkin, 2005; Karsunky et al., 2002; Velu et al., 2009). Several downstream targets of GFI1, such as ELA2 and CEBPe, have an established role in controlling hematopoiesis (Duan & Horwitz, 2003; Zhuang et al., 2006). In addition, Gfi1 controls transcription of the regulatory micro-RNAS miR-21 and miR-196B (Velu et al., 2009), as well as HoxA9, Pbx1, and Meis1 during normal myelopoiesis (Horman et al., 2009). Studies in Gfi1-deficient mice have demonstrated a role in TH2 (Zhu et al., 2002) and TH17 T cells (Zhu et al., 2009). Furthermore, Gfi1 has been implicated in B-cell differentiation (Rathinam & Klein, 2007) and control of autoimmunity (Rathinam et al., 2008). In light of these complex regulatory functions, multiple aberrant pathways may contribute to the phenotype of congenital neutropenia in patients with GFI1 mutations.

Congenital Neutropenia and Mutations in WAS

Loss-of-function WASp mutations cause Wiskott-Aldrich syndrome (Chapter 43). In contrast, gain-of-function mutations in WAS have been associated with a variant of congenital neutropenia (Devriendt et al., 2001). Affected patients carry mutations that affect the autoinhibitory structure of WASp, leading to conformational changes that induce increased actin polymerization. In addition to neutropenia, patients showed variable degrees of lymphopenia, reduced lymphocyte proliferation, and abrogated phagocyte activity (Ancliff et al., 2006). A large pedigree with multiple affected members has highlighted the clinical variability of patients harboring the same mutation (Beel et al., 2009). Interestingly, activating WASP mutations may also lead to myelodysplasia and overt leukemia (Ancliff et al., 2006; Beel & Vandenberghe, 2009). A similar phenotype could be reproduced in vitro using retroviral gene transfer of the mutant WASp (I294T) allele into hematopoietic stem cells (Moulding et al., 2007). This caused enhanced and delocalized actin polymerization throughout the cell, decreased proliferation, and increased apoptosis. Cells became binucleated, suggesting a failure of cytokinesis, and micronuclei were formed, indicative of genomic instability (Moulding et al., 2007). These elegant studies provided an interesting link between WASP as a regulator of the cytoskeleton and the control of cell division.

SCN WITH SYNDROMAL FEATURES

SCN with Lymphoid Deficiency and Inner Ear Hearing Loss—Reticular Dysgenesis

Reticular dysgenesis is an autosomal recessive form of early differentiation arrest in the myeloid lineage associated with severe combined immunodeficiency due to impaired lymphoid development (de Vall & Seynhaeve, 1959). In addition, affected patients suffer from sensorineural hearing loss (Small et al., 1999). Congenital neutropenia in reticular dysgenesis is not responsive to G-CSF (Bujan et al., 1993). Using a genome linkage analysis and candidate gene sequencing approach, mutations in adenylate kinase 2 (*AK2*) have been identified by two independent groups (Lagresle-Peyrou et al., 2009; Pannicke et al., 2009). AK2 is localized in the mitochondrial intermembrane space and may be important in mitochondrial energy metabolism and control of apoptosis via FADD and caspase 10 (Lee et al., 2007). The myeloid phenotype of AK2-deficient patients, in particular with respect to dissipation of the mitochondrial membrane potential, is very similar to HAX1-deficient patients, yet a link between HAX1 and AK2 remains unknown.

Disorders of Glycogen Metabolism

Two distinct but related disorders of neutrophil differentiation and viability have been linked to glucose metabolism. Glycogen storage disease type 1 is a well-known metabolic disorder leading to glycogen storage and hypoglycemia due to mutations in glucose-6-phosphatase (G6PC1). Patients with a related disease, glycogen storage disease type 1b, have metabolic features of glycogen storage disease type 1 and show in addition a phenotype of congenital neutropenia. Glycogenosis type 1b is caused by a mutations in *SLC37A4*, the gene encoding glucose-6-phosphate translocase (G6PT) (Gerin et al., 1997). Systemic complications such as liver adenomas, nephropathy, bone mineral density defect, polycystic ovaries, short stature, and Crohn's-like inflammatory bowel disease are also seen in this disorder, yet clear genotype–phenotype correlations could not be established (Melis et al., 2005). Patients with GSD-1b also show an increased prevalence of thyroid autoimmunity and hypothyroidism (Melis et al., 2007). A mouse model of G6PT deficiency has shed light on the pathophysiology. Murine GSD-Ib neutrophils exhibited increased production of ER chaperones and oxidative stress, leading to increased mitochondria-dependent apoptosis (Kim et al., 2008).

Recently, a novel genetic defect in the glucose-6-phosphate pathway has been discovered, caused by mutations in *G6PC3* (Boztug et al., 2009). In contrast to G6PC1 deficiency and G6PT deficiency, patients with G6PC3 deficiency present with congenital neutropenia and variable developmental disorders affecting the cardiovascular and/or urogenital system, such as atrial septum defect type II, cor triatriatum, or cryptorchidism and ureteral fistulas, respectively. Many patients show a characteristic visibility of subcutaneous veins, potentially due to decreased subcutaneous fat tissue.

G6PC3 is a ubiquitously expressed phosphatase located in the endoplasmic reticulum. In G6PC3-deficient cells, increased ER stress leads to the activation of the "unfolded protein response" and an increased susceptibility to apoptosis via decreased enzymatic activity of GSK3 beta (Boztug et al., 2009). Although functionally linked, G6PC3 deficiency and G6PT deficiency not only have distinct clinical phenotypes but are also different with respect to neutrophil function, suggesting that *G6PC3* and *SLC37A4* have nonredundant functions in their control of glucose metabolism in the ER.

Barth Syndrome

Barth syndrome is an X-linked dominant syndrome characterized by dilated cardiomyopathy, skeletal myopathy, growth deficiency, neutropenia, and 3-methylglutaconic aciduria (Barth et al., 1983; Kelley et al., 1991). The most common clinical presentation is cardiomyopathy in infancy. Bacterial infections secondary to neutropenia may be the first symptom, yet not all patients consistently show decreased neutrophil counts (Spencer et al., 2006).

The disease is caused by mutations in the ubiquitously expressed gene *TAZ*, which encodes tafazzin, a mitochondrial acyltransferase involved in cardiolipin metabolism (Bione et al., 1996). During cardiolipin maturation, phospholipase A removes one saturated acyl chain to generate monolyso-CL (MLCL), while tafazzin replaces it with an unsaturated acyl chain taken from phosphatidylcholine (PC), a process repeated until remodeling is complete (Xu et al., 2006). As a consequence, tafazzin-deficient cells show reduced mature cardiolipin levels and increased levels of monolysocardiolipin. Cardiolipin has been shown to participate in mitochondria-dependent apoptotic steps by serving as a "docking site" for tBid on the mitochondrial membrane (Lutter et al., 2000) and is required for Bax activation and mitochondrial outer membrane permeabilization (Kuwana et al., 2002).

Cardiolipin has been shown to participate in mitochondria-dependent apoptotic steps by serving as a "docking site" for tBid on the mitochondrial membrane and by anchoring caspase-8 for translocation into the mitochondrial membrane (Gonzalvez et al., 2008). Cardiolipin deficiency may therefore lead to decreased sensitivity to apoptosis, and tafazzin knockdown results in increased resistance to apoptosis. In neutrophils, a disassembly of protein complexes at the inner mitochondrial membrane is associated with exposure of phosphatidyl serine on the cell surface (Kuijpers et al., 2003).

Although phosphatidyl serine exposure is usually considered to be a marker for apoptosis, tafazzin-deficient neutrophils show no other apoptotic features and function normally, suggesting that neutropenia may not be caused by increased apoptosis of myeloid precursors or mature neutrophils. Since externalization of phophatidylserine enhances the recognition and engulfment of stressed cells by macrophages (Jitkaew et al., 2009), neutropenia in tafazzin deficiency may result from increased clearance of neutrophils by tissue macrophages (van Raam & Kuijpers, 2009). Thus, tafazzin deficiency, in contrast to HAX1 deficiency, may represent an interesting model system to study extrinsic mechanisms of neutrophil decay.

Congenital Neutropenia and Poikiloderma

A unique genodermatosis associating poikiloderma and neutropenia was reported by Clericuzio et al. in Navajo kindreds (Erickson, 1999). Two additional pedigrees have been published (Mostefai et al., 2008; Van Hove et al., 2005). This autosomal recessive syndrome is characterized by onset of a papular erythematous rash, pigment anomalies, telangiectasia, pachyonychia, hyperkeratosis, and variable degrees of congenital neutropenia. The molecular etiology remains unknown.

Congenital Neutropenia and Peripheral Neuropathy (Charcot-Marie-Tooth 2)

Charcot-Marie-Tooth (CMT) disease is a heterogeneous peripheral neuropathy disorder. CMT2, caused by monoallelic mutations in dynamin-2 (DNM2), has been associated with congenital neutropenia (Bitoun et al., 2008; Zuchner et al., 2005). DNM2 belongs to the family of large GTPases and is part of the cellular fusion-fission apparatus. DNM2 mutant proteins exhibit an altered conformation of the pleckstrin homology domain, thus affecting altered membrane dynamics. It remains to be shown how these aberrations lead to neutropenia.

Cohen Syndrome

Cohen et al. described an autosomal recessive syndrome including obesity, hypotonia, mental deficiency, craniofacial anomalies, and limb and spinal anomalies (Cohen et al., 1973). In the majority of Finnish patients the phenotype also included chorioretinal dystrophy and congenital neutropenia (Chandler et al., 2003; Norio et al., 1984).

Despite the phenotypic heterogeneity, Cohen syndrome is genetically homogeneous—all patients reported have a double mutation of *VPS13B* (*COH1*) (Kolehmainen et al., 2003). The human protein VPS13B is homologous to a yeast protein VPS13, which has a function in protein sorting and intracellular trafficking. However, there is no detailed explanation of how VPS13B deficiency causes the symptoms of Cohen syndrome, including neutropenia. Similar to other congenital neutropenia syndromes caused by aberrant vesicular trafficking (such as CHS, AP3 deficiency, p14 deficiency), Cohen syndrome is not associated with myeloid maturation arrest in the bone marrow.

NEUTROPENIA AND DISORDERS INVOLVING RIBOSOMAL PROTEINS

Infectious complications secondary to congenital neutropenia may be the first clinical presentation of a multisystem disorder characterized by defective ribosomal proteins: Shwachman-Diamond syndrome and cartilage-hair hypoplasia.

SHWACHMANN DIAMOND SYNDROME

Shwachmann-Diamond syndrome (SDS) was initially recognized as a complex syndrome associating exocrine pancreatic insufficiency and bone marrow dysfunction (Bodian et al., 1964; Shwachman et al., 1964). Clinically, most patients present with steatorrhea and failure to thrive. Neutropenia is the most common hematological feature, but anemia and thrombocytopenia may be seen as well. Myeloid cell development is characterized by increased sensitivity to apoptosis (Dror & Freedman, 2001). SDS predisposes to myelodysplasia and leukemia (Dror, 2005; Shimamura, 2006). SDS patients may also present with skeletal problems such as delayed growth and osteopenia (Makitie et al., 2004). Ninety percent of patients

with SDS show biallelic mutations in *SBDS*, a gene localized on chromosome 7q11 encoding a highly conserved protein with poorly defined functions (Boocock et al., 2003). Human SBDS localizes to the nucleolus in a cell-cycle–dependent manner (Austin et al., 2005) and is associated with ribosomal RNA (Ganapathi et al., 2007). Studies in the yeast SBDS ortholog Sdo1 revealed its importance for the release and recycling of the nucleolar shuttling factor Tif6 from pre-60S ribosomes, a key step in 60S maturation and translational activation of ribosomes (Menne et al., 2007). The detailed mechanism underlying the phenotype of neutropenia in SBDS-deficient cells remains enigmatic.

CARTILAGE-HAIR HYPOPLASIA

Cartilage-hair hypoplasia (CHH; McKusick et al., 1965) is a pleiotropic syndrome associating skeletal dysplasia, hypoplastic hair, gastrointestinal dysfunction, neutropenia, and lymphoid immunodeficiency (Lux et al., 1970). The syndrome was first described in the Amish community but is also prevalent in the Finnish population. Like SDS, CHH patients have a predisposition to bone marrow failure and cancer, in particular non-Hodgkin's lymphoma and basal cell carcinoma (Makitie et al., 1999; Taskinen et al., 2008; Thiel et al., 2007). CHH is caused by mutations in *RMRP*, encoding the RNA component of the endoribonuclease RNase MRP. RMRP has an important role in cleavage of RNA in mitochondrial DNA synthesis as well as in nucleolar cleavage of pre-rRNA (Ridanpaa et al., 2001). In view of the marked clinical variability, RMRP mutations should be considered when evaluating patients with combined immune deficiency, regardless of the presence of other manifestations (Kavadas et al., 2008).

Syndromes that Combine SCN or Neutrophil Dysfunction with Hypopigmentation

Some of the granules in neutrophils also belong to a more general class of lysosome-related organelles, sometimes called "secretory lysosomes" (Stinchcombe et al., 2004). Secretory lysosomes serve to sequester, transport, and secrete substances such as pigment and microbicidal peptides. Defects in secretory lysosomes may result in clinical phenotypes associating hypopigmentation and immunodeficiency. Four autosomal recessive traits have been described: Hermansky-Pudlak syndrome (HPS) type 2, Griscelli syndrome (GS) type 2, Chediak-Higashi syndrome (CHS), and p14 (MAPBPIP/ROBLD3) deficiency. Congenital neutropenia is consistently seen in HPS type 2 and p14 deficiency, whereas in GS type 2 and CHS (both discussed in Chapter 53), neutropenia may be absent or intermittent. The original syndrome described by Hermansky and Pudlak (1959) includes primarily hypopigmentation and prolonged bleeding times due to defective platelet granules. There are eight known human genes each of which is mutated in a different recessive form of HPS. Only HPS2, which is caused by a mutation in the gene *AP3B1*, includes a neutrophil dysfunction (Dell'Angelica et al., 1999). The AP3B1 protein is part of the heterotetrameric AP3 complex, which serves as an adaptor for endosomal transport networks. The cargo composition of AP3 in neutrophils is unknown, but Benson et al. suggested that AP3 may be a carrier for ELANE in both dogs and humans (Benson et al., 2003).

In addition to neutrophils, other cells of the immune system, such as NK cells (Enders et al., 2006; Fontana et al., 2006), NKT cells (Jung et al., 2006), and dendritic cells (Sugita et al., 2002), also appear to be affected in AP3-deficient patients.

Recently, a rare syndrome combining neutropenia, lymphoid immunodeficiency, and hypopigmentation was characterized in a single family with four affected children (Bohn et al., 2007). This syndrome is caused by a homozygous point mutation in the 3′ untranslated region of the gene that encodes p14 (MAPBPIP/ROBLD3) (Bohn et al., 2007). Residual p14/ROBLD3 protein likely explains why they can live, while p14-knockout mice are not viable (Teis et al., 2006). Other symptoms of p14/ROBLD3 deficiency include short stature, hypogammaglobulinemia (in particular low IgM serum levels), reduced numbers of B-cell subsets, and defective function of cytotoxic T cells, which may contribute to a general state of immunodeficiency. p14/ROBLD3-deficient neutrophils are defective in bacterial killing (Bohn et al., 2007). p14/ROBLD3 is an endosomal adaptor protein that acts as a scaffold when binding to MP1, enabling MP1 to participate in the ERK signaling cascade (Teis et al., 2002; Wunderlich et al., 2001).

Studies in AP3-deficient and p14/ROBDL3-deficient neutrophils illuminated the critical role of membrane trafficking for neutrophil homeostasis. However, further studies are required to explain the pathophysiology of neutropenia in these rare patients.

THERAPY OF SCN

The most important therapeutic goal in patients with congenital neutropenia is the reconstitution of adequate antibacterial host defense by neutrophil granulocytes. Recombinant human G-CSF (rh-G-CSF) is the first-line therapy of patients with SCN (Bonilla et al., 1989) G-CSF induces differentiation of neutrophil granulocytes and reduces apoptosis via multiple molecular effectors, including nicotinamide phosphoribosyltransferase (NAMPT) and NAD(+)-dependent sirtuin-1 activation (Skokowa et al., 2009b). Since the individual response to rh-G-CSF is quite variable, the dose should be titrated to achieve a protective neutrophil count of more than 1,000 neutrophils/microliter. Most patients respond to subcutaneous administration of rh-G-CSF, using starting doses of 5 microgram/kg/d. Patients who do not respond to even high-dose rh-G-CSF therapy (up to 50 to 100 microgram/kg) are considered poor responders. These individuals are candidates for allogeneic hematopoietic stem cell transplantation (HSCT) as a curative therapeutic strategy. Furthermore, patients developing myelodysplastic syndromes or leukemia should be candidates for allogeneic HSCT.

While allogeneic HSCT is currently the only curative therapy, there is hope that the molecular identification of underlying genetic defects may open horizons for specific

therapeutic approaches using transplantation of genetically modified autologous hematopoietic stem cells in the future.

CONGENITAL NEUTROPENIA AND PREDISPOSITION TO LEUKEMIA

Similar to several other inherited bone marrow failure syndromes, many subtypes of severe congenital neutropenia constitute premalignant conditions with an increased risk of clonal hematopoietic diseases such as myelodysplastic syndrome (MDS) and leukemia (Donadieu et al., 2005; Rosenberg et al., 2006, 2008). Most commonly, acute myeloid leukemia is seen, but patients with onset of acute lymphoid leukemia (Germeshausen et al., 2001a; Yetgin et al., 2008) or chronic myelomonocytic leukemia (Germeshausen et al., 2005) have also been described. The cumulative incidence for malignant transformation in congenital neutropenia after 10 years of G-CSF was 21 percent (Rosenberg et al., 2006). Recent data suggest that there may be no difference in leukemogenic risk for patients with mutations in HAX1 and ELA2 (Zeidler et al., 2009). In contrast, in certain subtypes of congenital neutropenia (e.g., G6PC3 deficiency, p14/ROBLD3 deficiency, AP3 deficiency), leukemic transformation has never been observed to date. Future studies will have to show whether the risk of clonal hematopoietic disorders depends on the genetic subtype.

Various acquired somatic mutations have been identified in hematopoietic cells of patients with SCN. Monoallelic mutations in the G-CSF receptor, encoded by the gene *CSF3R*, appear to play a key role in driving myelopoiesis. Nonsense mutations in *CSF3R*, truncating the distal cytoplasmic portion of the G-CSF receptor, are present in up to 40 percent of SCN patients and are strongly associated with the development of MDS/AML (Germeshausen et al., 2007). Of note, mutations in *CSF3R* may also be present prior to therapy with rh-G-CSF. The molecular pathophysiology of *CSF3R* mutations are under active investigation. Truncated mutants of the G-CSF receptor lack responsiveness to negative feedback loops (Hermans et al., 1999; van de Geijn et al., 2004). For example, deficient binding of SOCS3 may lead to misrouting of the G-CSF receptor and consecutively increased activation of STAT3/STAT5 (Irandoust et al., 2007; van de Geijn et al., 2004). Furthermore, deficient negative signaling via SHIP and cytokine-inducible Src homology 2 protein (CIS) (Hunter et al., 2004) may contribute to increased signaling via the G-CSF receptor. Using single-cell analysis of human bone marrow samples, Germeshausen et al. documented that CSF3R mutations are acquired in multipotent hematopoietic progenitor cells in congenital neutropenia patients and confer a survival advantage in myeloid progenitor cells (Germeshausen et al., 2009). Similar results were seen in a murine model system in which hematopoietic stem cells were transduced with vectors encoding a truncated G-CSF receptor. In competitive repopulation experiments, G-CSF-R^{mut} stem cells outgrew normal stem cells in a G-CSF–dependent manner (Liu et al., 2008). These studies document the pathogenic role of mutated G-CSF receptors as one critical event in the complex pathway of leukemogenesis. However, *CSF3R* mutations are not sufficient for driving leukemogenesis. This is illustrated by a recent report describing a large multigeneration pedigree in which an activating mutation in *CSF3R* has been found (Plo et al., 2009). Twelve of 16 members showed a point mutation in the G-CSF receptor (T617N), leading to chronic neutrophilia secondary to constitutive signaling via the G-CSF receptor. Only one patient developed an atypical myelodysplastic syndrome, suggesting that continuous activation via the G-CSF receptor may only represent a weak predisposing factor for clonal outgrowth.

Considering the risk of leukemogenesis in SCN patients, it has become clinical routine to screen for the onset of *CSF3R* mutations regularly (at yearly intervals). Mutations in *CSF3R* may be a first molecular signature preceding the development of a clonal disorder but do not justify the clinical diagnosis of MDS/AML. Additional molecular and cytogenetic aberrations commonly involve partial or complete losses of chromosome 7, activating RAS mutations, or abnormalities of chromosome 21 (Freedman et al., 2000). More comprehensive mutational profiling in 14 patients with AML/MDS on the basis of SCN revealed that the common mutations occurring in de novo AML (involving *FLT3, KIT, JAK2* genes) are not seen in leukemia cells arising in patients with SCN (Link et al., 2007). These findings suggest that the mechanisms governing leukemogenesis in SCN patients may be distinct from those molecular mechanisms controlling leukemogenesis in non-SCN patients.

CONCLUSIONS AND PERSPECTIVE

Despite the great progress that has been made in the field, many questions remain. First, patients with congenital neutropenia continue to suffer from severe and long-term complications, such as acute and chronic infections and subsequent organ damage. Of great clinical relevance, many patients are prone to develop myelodysplasia or leukemia. Second, in many patients the molecular etiology cannot yet be identified and the specific implications of defined genetic defects remain mysterious. At present, many published studies are Eurocentric and tend to neglect non-Caucasian ethnic groups, in whom other genetic defects may prevail. Third, the molecular pathophysiology of congenital neutropenia is far from being resolved—in particular, the epistatic relationships between defined genetic defects remain enigmatic. Finally, the only curative therapeutic approach consists of allogeneic HSCT, a procedure associated with severe morbidity and mortality. Novel gene-based therapies are only starting to be investigated in preclinical systems. Thus, a concerted global effort is needed to improve the diagnosis and therapy of patients with congenital neutropenia.

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52.

CHRONIC GRANULOMATOUS DISEASE

Dirk Roos, Steven M. Holland, and Taco W. Kuijpers

Chronic granulomatous disease (CGD) is an uncommon congenital immunodeficiency seen in approximately 1 in 250,000 individuals. It is caused by a profound defect in a burst of oxygen consumption that normally accompanies phagocytosis in all myeloid cells (neutrophils, eosinophils, monocytes, and macrophages). This "respiratory burst" involves the catalytic conversion of molecular oxygen to the oxygen free-radical superoxide (O_2^-), which in turn gives rise to hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and hydroxyl radical (•OH). These oxygen derivatives play a critical role in the killing of pathogenic bacteria and fungi. As a result of the failure to activate the respiratory burst in their phagocytes, the majority of CGD patients suffer from severe recurrent infections, the most common of which are pneumonia, lymphadenitis, cutaneous and hepatic abscesses, osteomyelitis, and septicemia. These severe infections usually become apparent during the first year of life and are caused predominantly by *Staphylococcus aureus*, *Aspergillus* species, enteric gram-negative bacteria, *Serratia marcescens*, *Burkholderia* (*Pseudomonas*) *cepacia* complex, or bacillus Calmette-Guérin (BCG). In addition, CGD patients have diffuse granulomas (presumably caused by microbes) that can become large enough to cause obstructive or painful symptoms in the esophagus, stomach, or biliary, urogenital, or pulmonary system.

While all CGD patients share the severe defect in the respiratory burst, there is substantial heterogeneity in the molecular mechanisms responsible. The enzyme that catalyzes the respiratory burst, NADPH oxidase, consists of at least five subunits (designated *phox* for phagocyte oxidase): gp91^{phox} and p22^{phox} (the two membrane-bound subunits of a low-potential flavocytochrome *b*, termed flavocytochrome b_{558}, that is the redox center of the oxidase) as well as three cytosolic oxidase components, p40^{phox}, p47^{phox}, and p67^{phox}. CGD is caused by a defect in any one of these five components. Mutations in the gp91^{phox} gene (*CYBB* on chromosome Xp21.1) cause the X-linked recessive form of the disease that affects about 70 percent of all CGD patients. As expected from the genetics, the overwhelming majority of X-linked patients are males. The remaining 30 percent of cases are inherited in an autosomal recessive manner in which males and females are equally affected. These patients have mutations in the genes encoding p40^{phox} (*NCF4* on chromosome 22q13.1; one patient known), p47^{phox} (*NCF1* on chromosome 7q11.23; about 20 percent of cases), p67^{phox} (*NCF2* on chromosome 1q25; about 5 percent of cases), or p22^{phox} (*CYBA* on chromosome 16q24; about 5 percent of cases). Deletions, insertions, splice-site defects, nonsense mutations, missense mutations, and, in rare cases, regulatory mutations have been identified in the five types of CGD at the molecular genetic level. With the exception of p47^{phox}-deficient patients, who are often homozygous for a GT deletion at the beginning of exon 2 in *NCF1*, many CGD patients have mutations unique to their families. The diversity of these mutations and the multiple genes affected provide an explanation for the genetic, biochemical, and clinical heterogeneity of CGD.

In two separate reports in 1957, Landing and Shirkey as well as Good et al. described a new syndrome of recurrent bacterial infections in boys that were associated with pigmented lipid-laden histiocytes in visceral organs, diffuse granulomas, and normal immunoglobulin levels (Berendes et al., 1957; Landing & Shirkey, 1957). The syndrome was originally referred to as "fatal granulomatous disease of childhood" because most patients died at early ages from severe infections that responded poorly to even the most aggressive therapy (Berendes et al., 1957; Bridges et al., 1959). Over the ensuing several decades, the prognosis for children afflicted with this rare disorder has steadily improved because of an increasing understanding of its molecular and clinical features and the

development of more active antibacterials and antifungals. Fatal granulomatous disease is now known by its more upbeat appellation, *chronic granulomatous disease* (CGD). What is remarkable about the early reports on CGD is the extent to which the clinical and genetic observations in a relatively small number of patients have held true; since then over 2,000 additional patients have been identified and, in numerous cases, reported in the literature.

Because the most striking hallmark of the early cases of CGD was unresolved bacterial and fungal infections, investigators were drawn to study the function of granulocytes from these patients. Indeed, patient neutrophils demonstrated markedly abnormal killing of organisms such as *S. aureus* and *Escherichia coli* in in-vitro assays (Quie et al., 1967). This defect was not due to an immunoglobulin or complement deficiency in patient serum, nor to an abnormality in neutrophil chemotaxis, ingestion, or degranulation. Patient neutrophils also contained normal levels of lysosomal proteins and degradative enzymes. The first clue to the underlying molecular defect came in 1959 in a seminal report by Sbarra and Karnovsky. It had been known since 1933 that neutrophils substantially increase their consumption of oxygen concomitant with phagocytosis of microbes (Baldridge & Gerard, 1933), and it had been assumed that this was due to increased energy metabolism. Sbarra and Karnovsky demonstrated, however, that the oxygen was not used for mitochondrial respiration but was instead utilized for the production of potentially microbicidal molecules such as hydrogen peroxide. This set the stage for the report in 1967 that CGD neutrophils did not undergo this respiratory burst when bacteria were ingested (Holmes et al., 1967). A subsequent report in 1973 identified the oxygen radical superoxide (O_2^-) as the initial product formed from the oxygen consumed in the respiratory burst and as the precursor of the other microbicidal oxygen derivatives (Babior et al., 1973). CGD neutrophils were found not to generate superoxide (Curnutte et al., 1974). The absence of the respiratory burst and its various products (such as superoxide and hydrogen peroxide) was then used as the defining characteristic of CGD and remains the cornerstone for its diagnosis today. Interestingly, one of the earliest assays used to measure respiratory burst activity in phagocytes, the nitroblue tetrazolium (NBT) dye reduction test, is still widely used for detecting CGD (Baehner and Nathan, 1968).

The NBT test also proved to be a powerful tool in defining the genetics of CGD. When used to stain neutrophils adherent to a microscope slide, the NBT "slide" test revealed that many of the mothers of affected males had two populations of cells: one that underwent a respiratory burst and stained positive, and another that failed to reduce the dye and therefore resembled the defective cells in the patient. While this finding indicated that CGD could be inherited in an X-linked recessive fashion, an autosomal recessive mode of inheritance was suggested by pedigrees with females suffering from a nearly identical syndrome of recurrent infections and absent respiratory burst, but normal respiratory burst in neutrophils from patients' mothers (Azimi et al., 1968; Quie et al., 1968).

Before this confusing genetic picture could be clarified, it was first necessary to identify the enzyme responsible for the respiratory burst. This proved to be a membrane-bound NADPH oxidase (Curnutte et al., 1975). An important breakthrough occurred in 1978 when Segal et al. found that a low-potential cytochrome *b* was undetectable in the neutrophils from four CGD patients (Segal et al., 1978). A multicenter European study of the prevalence of cytochrome *b* deficiency in a group of 27 CGD patients revealed that the cytochrome was undetectable in all 19 males who had X-linked CGD whereas it was present in normal levels in all 8 patients (7 females and 1 male) who appeared to have autosomal recessive inheritance (Segal et al., 1983). It thus appeared that cytochrome *b* (termed cytochrome b_{-245} or b_{558} to reflect its –245 mV midpoint potential or its 558-nm alpha absorption peak, respectively) was an important component of the NADPH oxidase and that X-linked CGD could be explained by its deficiency. The defect in autosomal recessive CGD remained unknown, and the picture was complicated by the identification of patients with autosomal recessive inheritance of the disease who had undetectable levels of cytochrome b_{558} (Weening et al., 1985b). Thus, at least three distinct forms of CGD appeared to exist—X-linked, cytochrome *b*-negative (X^0); autosomal recessive, cytochrome *b*-positive (A^+); and autosomal recessive, cytochrome *b*-negative (A^0)—as conclusively demonstrated by complementation studies in which monocytes from pairs of these three groups of patients were fused with each other and acquired NADPH oxidase activity (Hamers et al., 1984; Weening et al., 1985b).

One of the other major breakthroughs in unraveling the mystery of the genetic heterogeneity of CGD was the discovery in several laboratories of a cell-free system for activating NADPH oxidase (Bromberg & Pick, 1984; Curnutte, 1985; Heyneman & Vercauteren, 1984; McPhail et al., 1985). Studies with this system revealed that both the plasma membrane and cytosol fractions were required for oxidase activity. In the X-linked and the autosomal recessive forms of cytochrome b_{558} deficiency, it was the membrane fraction that was defective in the cell-free system (Curnutte, 1985; Curnutte et al., 1987), whereas in the autosomal recessive, cytochrome b_{558}-positive patients the cytosol was devoid of activity (Curnutte et al., 1988). The identities of the missing or defective proteins came to light in parallel with these findings through the convergence of several lines of investigation. First, Orkin et al. identified the gene affected in X-linked CGD, without knowledge of its protein product, through restriction fragment length polymorphism (RFLP) studies that localized the gene to Xp21 and the study of two patients with X-linked CGD who had small interstitial deletions within this region of the X chromosome (Baehner et al., 1986; Royer-Pokora et al., 1986). Second, Jesaitis, Parkos, et al. (Parkos et al., 1987) and Segal (1987) found that cytochrome b_{558} is a heterodimer composed of a heavily glycosylated 91-kDa subunit (termed β) and a nonglycosylated 22-kDa component (termed α). The 91-kDa subunit was found to be identical with the product of the X-CGD gene identified by Orkin et al. (termed *CYBB*) (Dinauer et al., 1987; Teahan et al., 1987), whereas the α subunit gene (termed *CYBA*) was localized to chromosome 16q24 and found to be defective in autosomal recessive, cytochrome b_{558}-negative patients (Dinauer et al., 1990;

Parkos et al., 1988). Finally, selected pairs of defective cytosols from patients with autosomal recessive, cytochrome b_{558}-positive CGD were found to complement each other in the cell-free activation system and to restore near-normal activity (Curnutte et al., 1989; Nunoi et al., 1988). Two cytosolic complementation groups were thus defined by this method, and the deficient proteins were identified as two novel myeloid-specific polypeptides—one that was 47 kDa in mass and the other 67 kDa (Leto et al., 1990; Nunoi et al., 1988; Volpp et al., 1988). In a cooperative study, the 47-kDa protein proved undetectable in 22 of 25 CGD patients with defective cytosols, whereas the 67-kDa polypeptide was absent in the remaining 3 patients (Clark et al., 1989). The gene encoding the 47-kDa component (termed *NCF1*) maps to 7q11.23 and the 67-kDa gene (termed *NCF2)* localizes to 1q25, findings consistent with the autosomal recessive mode of inheritance for each of these deficiencies in CGD (Francke et al., 1990).

A CGD-like syndrome caused by a defect in a third cytosolic component ($p40^{phox}$), in the activation mechanism of the NADPH oxidase (Rac2 deficiency), or by a shortage of substrate supply for the oxidase (glucose 6-phosphate dehydrogenase [G6PD] deficiency) has to be considered for differential diagnosis (see below).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Given the key role that the products of the respiratory burst plays in host defense, it is not surprising that patients with CGD suffer from a variety of recurrent bacterial and fungal infections. These infections occur most commonly in organs in contact with the outside world—the lungs, gastrointestinal tract, and skin, as well as the lymph nodes that drain these structures. Because of both contiguous and hematogenous spread of infection, a wide range of other organs can be affected, most notably the liver, bones, kidneys, and brain.

In approximately two thirds of patients, the first symptoms of CGD appear during the first year of life in the form of infections, dermatitis (sometimes seen at birth), gastrointestinal complications (obstruction or intermittent bloody diarrhea due to colitis) (Johnston & Newman, 1977), and a failure to thrive. The clinical picture can be quite variable, with some infants suffering from several of these complications, whereas others appear far less ill. In some cases, the presenting symptoms of CGD can be mistaken for pyloric stenosis, food or milk allergy, or iron-deficiency anemia. In general, infants with X-linked CGD tend to present earlier and with more dramatic symptoms than infants with the autosomal recessive forms of the disease, especially $p47^{phox}$ deficiency (Gallin et al., 1991; Jones et al., 2008; Margolis et al., 1990; Martire et al., 2008; Van den Berg et al., 2009; Weening et al., 1985a; Winkelstein et al., 2000). In a small but noteworthy subset of patients, the diagnosis of CGD is not made until the teenage or adult years because of the mildness or lack of clinical symptoms, as discussed below.

The full spectrum of CGD is summarized in Table 52.1. Despite the rarity of CGD, a number of investigators have either reviewed or assembled large series of patients from the United States, Europe, and Japan (Cohen et al., 1981; Forrest et al., 1988; Gallin et al., 1983; Hayakawa et al., 1985; Hitzig and Seger, 1983; Johnston & Newman, 1977; Jones et al., 2008; Martire et al., 2008; Mouy et al., 1989; Tauber et al., 1983; Van den Berg et al., 2009; Winkelstein et al., 2000). The approximately 1,500 patients represented by these studies provide a striking picture of the multiple manifestations of the disease. As shown in Table 52.1, *S. aureus*, *Aspergillus* species, enteric gram-negative bacteria (including *S. marcescens* and various *Salmonella* species), and *B. cepacia* complex (previously termed *P. cepacia*) are the most frequently encountered pathogens. Most CGD pathogens share the property of producing catalase; as such, they degrade the hydrogen peroxide that they themselves generate within the phagolysosome, thereby failing to complement the peroxide-starved CGD phagocytes. It was therefore suggested that catalase-negative organisms, by supplying the CGD phagocytes with microbial hydrogen peroxide, might complement the hydrogen peroxide deficit in CGD phagocytes, thus inducing killing of the microbes themselves (Mandell & Hook, 1969). Catalase production was thus thought to be an important microbial pathogenicity factor in CGD. However, this hypothesis must be viewed in the context that the majority of all pathogens contain catalase (with the important exception of streptococci). This view has been further challenged by the retained virulence of *Aspergillus* and staphylococci rendered genetically deficient for catalase production (Chang et al., 1998; Messina et al., 2002).

Pneumonia is the most common type of infection encountered in CGD in all age groups and is typically caused by *S. aureus*, *Aspergillus* species, *B. cepacia*, and enteric gram-negative bacteria (Fig. 52.1). If the pneumonia is already severe or the patient fails to respond promptly to the empiric regimen, more invasive approaches are warranted to establish the diagnosis, such as needle biopsy, bronchoscopy, or open lung biopsy. Rarer pathogens requiring special types of treatment (for example, *Nocardia*) are usually only identified by these more aggressive approaches. One of the more problematic pulmonary pathogens is *B. cepacia* complex (previously divided into genomvars, now recognized as multiple species, including *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. vietnamiensis*, *B. stabilis*, *B. ambifaria*, *B. dolosa*, *B. anthina*, and *B. pyrrocinia*), as well as the closely related organisms *B. gladioli*, *B. mallei*, *B. pseudomallei*, and *B. pickettii.* These bacteria are unusually virulent in CGD patients and have come to be identified as one of the causes of fatal pneumonias in these patients (Speert et al., 1994). Contributing to this mortality is the fact that many strains of *Burkholderia* are not adequately eliminated during empiric treatment of CGD infections, because *Burkholderia* species are generally resistant to aminoglycosides and some also to ceftazidime. More importantly, cultures of *Burkholderia* may grow slowly, resulting in late diagnosis.

Aspergillus and other fungal infections of the lung also pose difficult challenges because they typically require prolonged treatment (3 to 6 months). If possible, it is important to determine the source of the *Aspergillus* in the patient's environment so that the risk of reexposure can be minimized, such

Table 52.1 INFECTIONS, CHRONIC CONDITIONS, AND ORGANISMS ASSOCIATED WITH CGD*

INFECTIONS	PERCENT OF INFECTIONS	CONDITION	PERCENT OF CASES	INFECTING ORGANISMS	PERCENT OF ISOLATES
Pneumonia	70–80	Lymphadenopathy	98	*Staphylococcus aureus*	30–50
Lymphadenitis†	60–80	Hypergammaglobulinemia	60–90	*Escherichia coli*	5–10
Cutaneous infections/impetigo†	60–70	Hepatosplenomegaly	50–90	*Aspergillus* species	10–20
Hepatic/perihepatic abscesses†	30–40	Splenomegaly	60–80	*Salmonella* species	5–10
Osteomyelitis	15–30	Anemia of chronic disease	40–60	*Klebsiella* species	5–10
Septicemia	10–20	Underweight	70	*Burkholderia cepacia*	5–10
Otitis media†	15–20	Chronic diarrhea	20–60	*Staphylococcus epidermidis*	5
Conjunctivitis	10–20	Short stature	50	*Serratia marcescens*	5–10
Enteric infections	5–15	Gingivitis	50	*Enterobacter* species	3
Urinary tract infections/pyelonephritis	5–15	Dermatitis	35	*Streptococcus* species	4
Sinusitis	<10	Chorioretinitis	20–35	*Proteus* species	3
Renal/perinephric abscesses	<10	Hydronephrosis	10–25	*Candida albicans*	3
Brain abscesses	<5	Ulcerative stomatitis	5–25	*Nocardia* species	2
Pericarditis	<5	Pulmonary fibrosis	<10	*Haemophilus influenzae*	1
Meningitis	<5	Esophagitis	<10	Bacillus Calmette-Guérin	<1
		Gastric antral narrowing Granulomatous	<10	*Mycobacterium* species	<1
		ileocolitis Granulomatous cystitis Glomerulonephritis	<10	*Chromobacterium violaceum*	<1
			<10	*Francisella philomiragia*	<1
		Discoid lupus erythematosus	<10	*Candida glabrata*	<1
			<10	*Actinomyces*	<1
				Granulibacter bethesdensis	<1
				Leishmania species	<1

*The relative frequencies of different types of infection in CGD are estimated from data pooled from several large series of patients in the United States, Europe, and Japan (Cohen et al., 1981; Forrest et al., 1988; Gallin et al., 1983; Hayakawa et al., 1985; Hitzig & Seger, 1983; Johnston & Newman, 1977; Jones et al., 2008; Martire et al., 2008; Mouy et al., 1989; Tauber et al., 1983; Winkelstein et al., 2000; Van den Berg et al., 2009). These series encompass approximately 1,500 CGD patients after accounting for overlap between reports. The list of infecting organisms is also arranged according to data in these reports and is not paired with the entries in the first column.

†These infections are most frequently seen at the time of presentation.

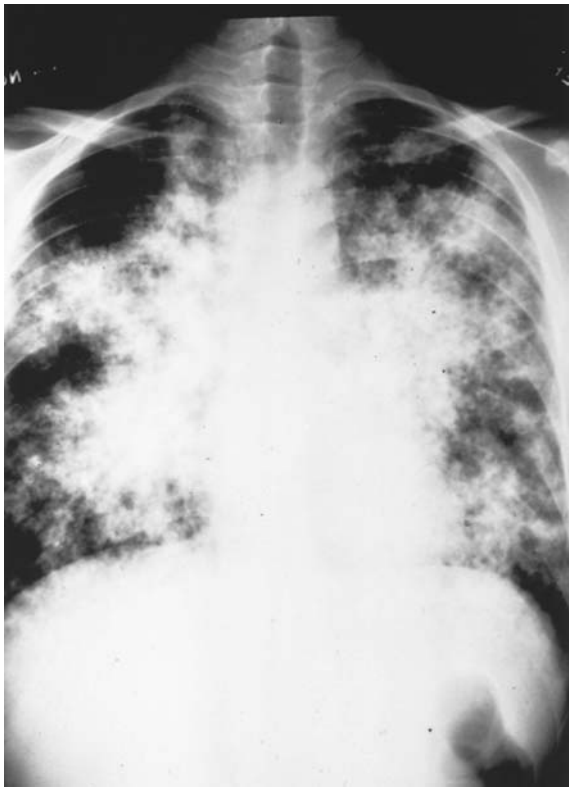

Figure 52.1 Chest X-ray of a male patient with CGD and fatal *Aspergillus* infection.

as exposure to dirt, manure, leaves, mulch, or other decaying organic matter.

Cutaneous abscesses and lymphadenitis represent the next most common types of infection in CGD and are typically caused by *S. aureus*, followed by various gram-negative organisms, including *B. cepacia* complex and *S. marcescens* (Color Plate 52.I). These infections may respond slowly to antimicrobial therapy and often require incision and drainage for resolution. Recurrent impetigo, frequently in the perinasal area and caused by *S. aureus*, usually requires prolonged courses of oral and topical antibiotics to clear. Hepatic (and perihepatic) abscesses are also quite common in CGD and are typically caused by *S. aureus.* Patients usually present with fever, malaise, and weight loss. Liver function tests are often normal. Resolution may require surgical drainage or excision of the lesion as well as several months of parenteral antibiotics. A recent report cites combined steroid and antibiotic therapy for staphylococcal liver abscesses in CGD patients (Leiding et al., 2011). Osteomyelitis is another important infection in CGD and can arise from hematogenous spread of organisms (*S. aureus*, *Salmonella* spp., *S. marcescens*) or contiguous invasion of bone, typically seen with non-*fumigatus Aspergillus* pneumonia, such as *Aspergillus nidulans* spreading to the ribs or vertebral bodies (Segal et al., 1998). Perirectal abscesses are also common in CGD patients and once formed can persist for years despite aggressive antimicrobial therapy and fastidious local care. Other important but less commonly seen infections in CGD are summarized in Table 52.1.

Not all encounters with microorganisms in CGD result in overt pyogenic infections, as stalemates may develop between the pathogen and the patient's leukocytes. In these cases, chronic inflammatory cell reactions consisting of lymphocytes and histiocytes develop, which can then organize to form granulomas, one of the hallmarks of CGD and causing various clinical symptoms of obstruction.

Another important type of chronic inflammation in CGD is a form of inflammatory bowel disease that closely resembles Crohn's disease and affects 10 to 43 percent of patients (Marciano et al., 2004a). While the colon is typically involved, the ileum and other parts of the gastrointestinal tract can be affected. The symptoms can range from mild diarrhea to a debilitating syndrome of bloody diarrhea and malabsorption that can even necessitate colectomy (Ament & Ochs, 1973). Other types of chronic inflammation include gingivitis, chorioretinitis or uveitis, glomerulonephritis, and—rarely—destructive white matter lesions in the brain (Table 52.1) (Frifelt et al., 1985; Hadfield et al., 1991; Martyn et al., 1972) and discoid or even systemic lupus erythematosus (Manzi et al., 1991; Sillevis Smitt et al., 1990a). The eye problems are only rarely of clinical importance or progressive of nature (Buggage et al., 2006; Goldblatt et al., 1999; Palestine et al., 1983).

Lymphadenopathy, hepatosplenomegaly, and eczematoid dermatitis are also common features of CGD and are most frequently encountered in infants and children. Children with CGD are typically anemic, with hemoglobin levels in the 8 to 10 g/dL range and with microcytic erythrocyte indices. The anemia is most likely due to the chronic disease state and, not surprisingly, responds poorly to oral iron therapy. However, iron absorption is also impaired in CGD, perhaps reflecting inflammatory bowel disease. Some X-linked CGD patients have McLeod syndrome, a mild hemolytic anemia characterized by acanthocytosis and weak or absent expression of Kell antigens (Marsh & Redman, 1987). McLeod syndrome is caused by interstitial deletions in the X chromosome that affect both the gene for gp91^{phox} (*CYBB*) and the nearby *Xk* gene that encodes a 37-kDa erythrocyte membrane protein necessary for expression of Kell antigens.

Carriers of CGD, whether of the X-linked form or any of the three main autosomal recessive forms, are usually asymptomatic, with three important exceptions. First, roughly half of X-linked carriers have either recurrent stomatitis or moderately severe aphthous ulceration or both. Second, in a small percentage of X-linked carriers, there can be an unusually high degree of inactivation of the normal X chromosome in the myeloid cells. If the circulating neutrophil population is skewed to the point that fewer than 10 percent of the cells function, then the carrier has an increased risk of infection (usually mild) (Curnutte et al., 1992; Mills et al., 1980). Third, approximately one quarter of X-linked carriers develop discoid lupus erythematosus, characterized by discoid skin lesions on the sun-exposed regions such as the face, shoulders, arms, upper chest, and back. This syndrome typically becomes apparent in the second decade of life, can range from mild (common) to fairly intense, usually responds well to hydroxychloroquine, and does not appear to progress to systemic lupus erythematosus (Brandrup et al., 1981; Manzi et al., 1991; Schaller, 1972;

Sillevis Smitt et al., 1990b; Yeaman et al., 1992). Discoid lupus erythematosus has not been associated with the carrier state in any of the autosomal recessive forms of CGD. The underlying mechanism responsible for discoid lupus in X-linked CGD carriers is not known, although it has been hypothesized that autoantibodies to antigens from incompletely destroyed microbes (derived from the defective subpopulation of neutrophils) may be responsible (Manzi et al., 1991).

COMPOSITION, ACTIVATION, AND FUNCTION OF THE NADPH OXIDASE

COMPOSITION OF THE NADPH OXIDASE

The phagocyte NADPH oxidase consists of a membrane-bound catalytic subunit and a number of activity-regulating components that reside in the cytosol of resting phagocytes but translocate to the membrane upon cell activation (Fig. 52.2). In this way, the activity of this potent enzyme is restricted to the time when and the site where it is needed. The membrane-bound catalytic subunit is the flavohemeprotein gp91^{phox}, which is stabilized in a 1:1 complex with the transmembrane p22^{phox} protein (Table 52.2) (Huang et al., 1995; Wallach & Segal, 1996). The complex together is called flavocytochrome b_{558}. The N-terminal half of gp91^{phox} contains six hydrophobic regions that serve as membrane-spanning domains and three N-linked glycosylation sites (Wallach & Segal, 1997) (Fig. 52.3). This part of gp91^{phox} contains two heme moieties, each located between two histidines in transmembrane domains of the protein (Fig. 52.3) (Biberstine-Kinkade et al., 2001; Cross et al., 1995; Finegold et al., 1996; Yu et al., 1998). This part of gp91^{phox} also appears to be involved in the interaction with p22^{phox}. The C-terminal part of gp91^{phox} is hydrophilic, probably cytosolic, and contains one FAD group. The location of the putative FAD binding domain has been deduced from sequence homology between the C-terminal half of gp91^{phox} and the ferridoxin NADP reductase flavoenzyme family (Leusen et al., 2000; Rotrosen et al., 1992; Segal et al., 1992; Sumimoto et al., 1992). In a similar way, the position of the putative NADPH binding domain has been mapped on gp91^{phox} (Fig. 52.3). Like gp91^{phox}, the N-terminal half of p22^{phox} contains hydrophobic helices that may be transmembrane domains, and the C-terminal half is more hydrophilic.

The cytosolic NADPH oxidase components p47^{phox} and p67^{phox} each contain two src homology region 3 (SH3) motifs, known to interact with proline-rich regions in the same or in other proteins (Table 52.2, Fig. 52.4). Both of these cytosolic components also contain at least one such proline-rich region. The SH3–proline interactions may be involved both in keeping p47^{phox} and p67^{phox} in an inactive conformation in resting cells (by mutual interaction) and in activating the NADPH oxidase enzyme by promoting interactions between the cytosolic components and flavocytochrome b_{558} (De Mendez et al., 1994; Finan et al., 1994; Leto et al., 1994; Leusen et al., 1994a, 1994b, 1995; Sumimoto et al., 1994). The p67^{phox} protein also contains four tetra-tricopeptide (TPR) regions

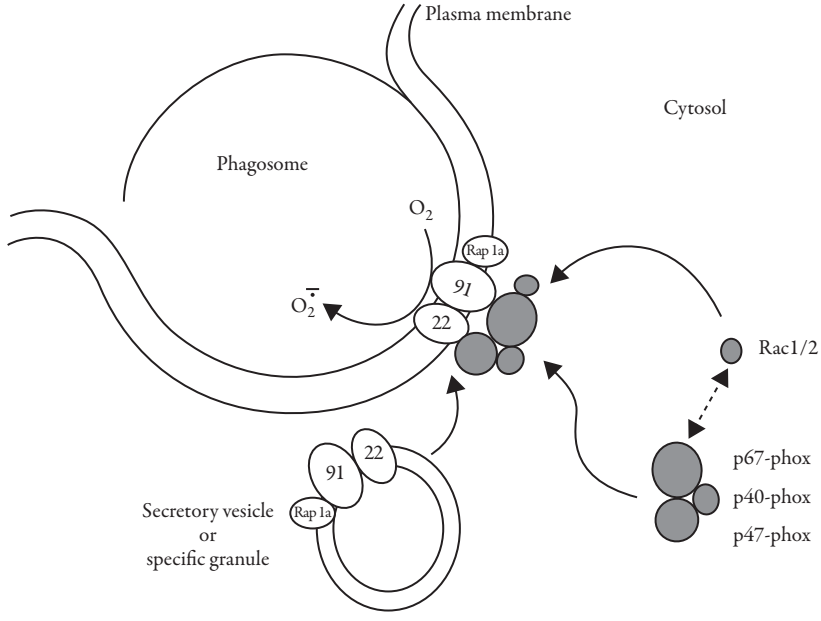

Figure 52.2 Activation of the NADPH oxidase. The membrane-bound NADPH-oxidase components gp91^{phox} and p22^{phox} and the small GTP-binding protein Rap1A are located in resting neutrophils in the membranes of specific granules and secretory vesicles. During cell activation (e.g., after binding of opsonized microorganisms to surface receptors on the neutrophils), these organelles fuse with the plasma membrane, thus leading to upregulation of the expression of these components on the neutrophil surface, especially in plasma membrane areas surrounding phagocytosed material (phagosomes). Concomitantly, the cytosolic NADPH-oxidase components p40^{phox}, p47^{phox}, and p67^{phox} translocate to the cytochrome b_{558} subunits (gp91^{phox} and p22^{phox}) and induce a conformational change in gp91^{phox} that allows NADPH to bind to gp91^{phox} and donate its electrons to the FAD moiety in this subunit. The cytosolic small GTP-binding proteins Rac1 (in macrophages) and Rac2 (in neutrophils) regulate the translocation process by binding to p67^{phox}, to gp91^{phox}, and to the plasma membrane.

Table 52.2 PROPERTIES OF THE PHAGOCYTE RESPIRATORY BURST OXIDASE (*PHOX*) COMPONENTS

	GP91^{phox}	*P22*^{phox}	*P47*^{phox}	*P67*^{phox}	*P40*^{phox}
SYNONYMS	β-CHAIN HEAVY CHAIN NOX-2	α-CHAIN LIGHT CHAIN	NCF-1	NCF-2	NCF-4
Amino acids	570	195	390	526	339
Molecular weight (kDa)					
Predicted	65.0	20.9	44.6	60.9	39.0
By PAGE	70–91	22	47	67	40
Glycosylation	Yes (N-linked)	No	No	No	No
Phosphorylation	Low level	Yes	Yes	Low level	Low level
PI	9.7	10.0	9.5	5.8	6.4
mRNA(kb)	4.7	0.8	1.4	2.4	1.4
Gene locus	*CYBB* Xp21.1	*CYBA* 16q24	*NCF1* 7q11.23	*NCF2* 1q25	*NCF4* 22q13.1
Exons/span	13/30 kb	6/8.5 kb	11/15 kb	16/40 kb	10/18 kb
Cellular location in resting neutrophils	Specific granule membrane; plasma membrane	Specific granule membrane; plasma membrane	Cytosol; cytoskeleton	Cytosol; cytoskeleton	Cytosol; cytoskeleton
Level in neutrophils (pmoles/10^6 cells)	3.3–5.3	3.3–5.3	3.3	1.2	1.2
Tissue specificity	Myeloid, low levels in mesangial cells, endothelial cells, and B lymphocytes	mRNA in all cells tested, protein only in myeloid and endothelial cells	Myeloid cells and B lymphocytes	Myeloid cells and B lymphocytes	Myeloid and other hematopoietic cells
Functional domains	Binding sites for cytosolic oxidase components; heme, FAD, and NADPH binding domains	Pro-rich domain that binds p47^{phox}	Nine Ser-P sites; 2 SH3 domains; Pro-rich domains; 1 PX domain	Two SH3 domains; 4 TTP regions; 1 PB1 domain; Pro-rich domains	One SH3 domain, 1 PX domain, 1 OPR domain
Homologies	Ferredoxin-NADP$^+$ reductase (FNR)* (weak homology)	Polypeptide I of cytochrome *c* oxidase	SH3 domain of *src*, p47^{phox}, and p67^{phox}; PX domain of many proteins†; homology to p40^{phox} N terminus	SH3 domain of *src*, p40^{phox}, and p47^{phox}; TTP regions of many proteins†; PBI domain of budding yeast protein Bem1p	SH3 domains of *src* and p67^{phox}; OPR domain of Cdc24p, MEK5, and Zip; PX domain of many proteins†; homology to p47^{phox} N terminus
GenBank Accession No.	X04011**	M21186, J03774	M25665, M26193	M32011	U50720-U50729

ABBREVIATIONS: *CYBA*, cytochrome-*b* alpha; *CYBB*, cytochrome-*b* beta; FNR, ferredoxin-NADP reductase; *NCF*, neutrophil cytosol factor; OPR, octicosa peptide repeat; PAGE, polyacrylamide gel electrophoresis; PB1, Phox and Bem-1 domain; phox, *ph*agocyte *ox*idase component; Pro, proline; PX, phox homology; Ser-P, serine phosphorylation; SH3, *src* homology domain 3; TTP, tetratricopeptide.

*Weak homology to both the NADPH and FAD binding domains in the FNR family.

†PX domain homology with kinesins, phospholipases, PI3-kinases, protein kinases, SNAREs, and sorting nexins.

†TTP region homology with subunits of anaphase-promoting complex, hsp90-binding immunophilins, transcription factors, PKR protein kinase inhibitor, and peroxisomal and mitochondrial import proteins.

**This GenBank accession number refers to the sequence as originally published. The complete corrected sequence (encoding an additional 64 amino acids) has not been deposited in GenBank.

in its C terminus, which form binding regions for the small GTPases Rac1 or Rac2; this constitutes another activation mechanism of the phagocyte NADPH oxidase (Koga et al., 1999; Nisimoto et al., 1997). A third cytosolic component, called p40^{phox}, with N-terminal homology to p47^{phox} and one SH3 domain resembling that of p67^{phox}, is possibly involved in stabilization of the p47^{phox}/p67^{phox} complex in resting cells and in facilitating membrane recruitment of this complex during oxidase activation (Table 52.2, Fig. 52.4) (Fuchs et al., 1995; Kuribayashi et al., 2002). Both p47^{phox} and p40^{phox} contain a so-called PX (phox homology) region that can bind to phosphatidyl phosphoinositides in the plasma membrane as yet another interaction to stabilize the active NADPH oxidase complex (Ellson et al., 2001; Kanai et al., 2001). The PX domain in p47^{phox} contains a PXXP motif, a proline-rich region that interacts with both SH3 groups of p47^{phox} in the

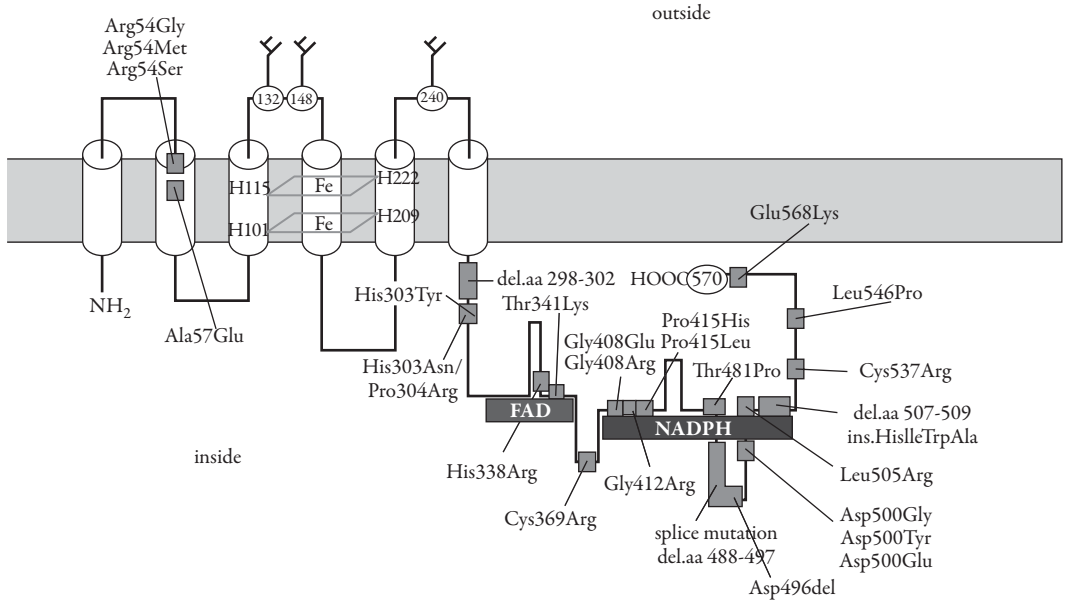

Figure 52.3 Schematic picture of gp91^{phox} and X91^{+} CGD mutations. The hydrophobic regions of gp91^{phox} that serve as membrane-spanning domains are shown as cylinders, while the asparagines that are N-glycosylated are indicated by the codon number and a carbohydrate tree. The heme-binding histidines are located in the third and fifth transmembrane regions. The putative FAD and NADPH binding regions are also indicated, as are the mutations that lead to the X91^{+} phenotype of CGD. The loop of 20 amino acids that covers the cleft of the NADPH binding region in resting cells is indicated in the lower right corner of the picture.

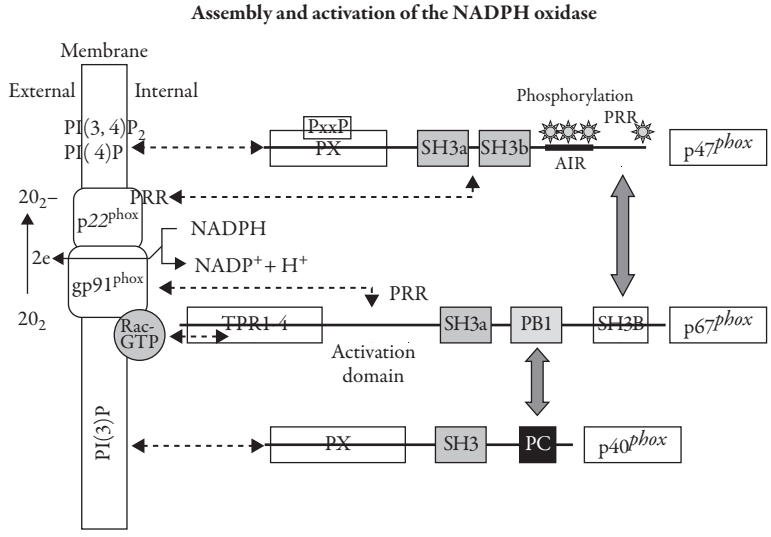

Figure 52.4 Assembly and activation of the NADPH oxidase. In the resting state, the three cytosolic NADPH oxidase subunits p47^{phox}, p67^{phox}, and p40^{phox} form a trimeric complex in the cytosol in which p47^{phox} is folded, kept together by interactions between the PXXP region in its PX domain with the two SH3 regions, and by the C-terminal proline-rich region (PRR) also interacting with the two SH3 regions (Groemping et al., 2003). The PRR in p47^{phox} is supposed to also interact with the SH3 region of p40^{phox} in resting cells, which in turn interacts with the PB1 region of p67^{phox} via a PC motif (*bottom arrow*). Phosphorylation of several serines (*stars*) in the polybasic region of p47^{phox} in activated phagocytes, between the C-terminal SH3 region (SH3b) and the PRR (the auto-inhibitory region [AIR]), causes unfolding of p47^{phox} and thus exposure of the SH3 regions in this protein (Fuchs et al., 1995; Leto et al., 1994). These p47^{phox} SH3 regions are then free to interact with the PRR in p22^{phox}, and the PX region of p47^{phox} is now free to interact with PI(3,4)P_2 and PI(4)P in the plasma membrane around the flavocytochrome b_{558}. Moreover, unfolding of p47^{phox} also leads to release of p40^{phox} from p47^{phox}, leaving the PRR in p47^{phox} free to associate with the carboxy SH3 domain of p67^{phox} in the oxidase complex assembled at the membrane (*top arrow*). P40^{phox} can now bind with its PX region to PI(3)P in the membrane, and p67^{phox} can interact through its TPR regions with Rac-GTP, which is associated through its insert domain with gp91^{phox} and with its lipid tail in the membrane. At the same time, the interaction between p40^{phox} and p67^{phox} (*bottom arrow*) is broken and p67^{phox} associates with its activation domain with gp91^{phox}; this latter interaction is essential for electron flow within the flavocytochrome. All new interactions in activated cells are indicated as broken arrows.

resting state of this protein (Hiroaki et al., 2001) (Fig. 52.4). P40^{phox} also contains in its C terminus an octicosa peptide repeat (OPR), also called PC (Phox Cdc24p) domain, which is involved in binding to a so-called PB1 (Phox and Bem-1) domain in p67^{phox} (Ito et al., 2001; Nakamura et al., 1998; Ponting, 1996). The regions within the cytoplasmic oxidase components that interact with each other or with flavocytochrome b_{558} are partially known from studying natural mutations (Dinauer et al., 1991; Leto et al., 1994; Leusen et al., 1994a, 1994b, 2000; Sumimoto et al., 1994). More detailed information has been gained from analyzing peptides in the two-hybrid system (Fuchs et al., 1995) or in a phage display system (DeLeo et al., 1995a, 1995b), and from peptide walking (Joseph & Pick, 1995; Morozov et al., 1998).

ACTIVATION OF THE NADPH OXIDASE

In the inactive state of the NADPH oxidase in quiescent phagocytes, the flavohemeprotein gp91^{phox} is inaccessible for its substrate NADPH because a stretch of 20 amino acids (residues 484–504) lies over the nucleotide-binding cleft (Fig. 52.5) (Taylor et al., 1993). Interaction of p47^{phox} and p67^{phox} with flavocytochrome b_{558} results in movement of this 20–amino acid loop and hence provides NADPH with access to its binding site in gp91^{phox}. The translocation of the cytosolic NADPH oxidase components to the flavocytochrome is initiated by serine phosphorylation of p47^{phox} (and of p67^{phox} and p40^{phox}) during phagocyte activation (Bolscher et al., 1989; Bouin et al., 1998; Dusi and Rossi, 1993; Forbes et al., 1999; Okamura et al., 1988; Segal et al., 1985) (Fig. 52.4). For more details of this process see the legend to Figure 52.4. One proline-rich region in p22^{phox} involved in binding an SH3 region in p47^{phox} has been identified from the mutation in this proline-rich region in a CGD patient (Leto et al., 1994; Leusen et al., 1994a; Sumimoto et al., 1994). It is not yet known which protein kinases are responsible for the phosphorylation of these oxidase components, nor how these kinases are activated. Possibly, protein kinase C-β plays a role in this respect (El Benna et al., 1996; Korchak et al., 1998; Reeves et al., 1999).

Positioning of the cytosolic oxidase components in the membrane is facilitated by the formation of phosphatidylinositol (PtdIns) species phosphorylated at the inositol-3 position in the membrane by phosphoinositide-3-OH kinase [PI(3)K] during neutrophil activation. The PX domain of p40^{phox} binds to PtdIns(3)P and the PX domain of p47^{phox} binds to PtdIns(3,4)P_2 (Kanai et al., 2001). Charge interactions between gp91^{phox} and the cytosolic components may also play a role in the formation of the oxidase complex, because several substitutions of amino acids in gp91^{phox} for amino acids with a different charge lead to stable expression of mutant gp91^{phox} molecules that no longer support translocation of cytosolic components (Leusen et al., 1994b, 2000). The proper positioning and stabilization of the oxidase complex may also depend on binding of the cytosolic components to cytoskeletal proteins, such as coronin, actin, moesin, and calprotectin, the heterodimer of the calcium-binding proteins S100A8/A9 (Doussière et al., 2002; Grogan et al., 1997; Tamura et al., 2000; Wientjes et al., 2001). In addition, the myeloid-related proteins MRP8 and MRP14 appear to be involved in the conformational change induced in gp91^{phox} during phagocyte activation, in synergy with p47^{phox} and p67^{phox} (Berthier et al., 2003). Finally, cPLA2-generated arachidonic acid is an essential cofactor in NADPH oxidase activation in intact or permeabilized cells, but the mechanism of this phenomenon is unknown (Dana et al., 1998). It must be stressed that the assembled NADPH oxidase complex is not a stable polypeptide complex: there is continuous binding to and release of cytosolic oxidase components from the membrane-bound components, but it is unknown whether electron transfer from NADPH to oxygen requires the actual presence of the cytosolic components in the complex (Van Bruggen et al., 2004).

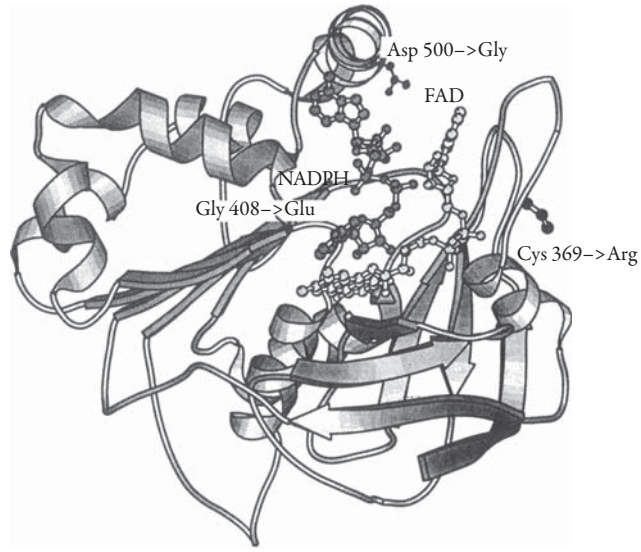

Figure 52.5 Three-dimensional model of the C-terminal, cytosolic part of gp91^{phox}. This figure shows a model of the cytosolic portion of gp91^{phox} based on that for spinach ferridoxin NADP reductase (Karplus et al., 1991), as described by Taylor et al. (1993). The figure was kindly provided by Dr. Nicholas Keep from Prof. Segal's group (London, UK). The protein is shown in the activated, NADPH-bound state. The 20–amino acid coil covering the cleft over the NADPH binding region in the active protein is now drawn aside (on top of the picture). In this coil, the position of Asp500 is indicated. Cys369 is located in another protein loop exposed to the cytosol, but Gly408 is buried in the protein between these two loops. Mutations Asp500Gly, Cys369Arg, and Gly408Glu lead to the X91^{+} CGD phenotype with defective translocation of cytosolic NADPH oxidase components.

Several low-molecular-weight GTP-binding proteins are involved in the NADPH oxidase activation (Table 52.3) (Abo et al., 1991; Knaus et al., 1991). One of these is p21rac, of which two subtypes exist in phagocytes—Rac1 (mainly in macrophages) and Rac2 (mainly in neutrophils) (Dusi et al., 1995). During neutrophil activation, these GTP-binding proteins change from an inactive, GDP-bound state into an active, GTP-bound state, a process that is regulated by guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Two Rac-activating GEFs have been identified in phagocytes: P-Rex1 and Vav1. P-Rex1 is synergistically activated by PtdIns(3, 4, 5)P_3 and the βγ subunits of trimeric G proteins associated with seven-span membrane receptors

(Welch et al., 2002). This indicates that P-Rex1 in neutrophils is under direct control of ligand binding to these receptors, but this was proven only for C5a. Vav1 is activated by tyrosine phosphorylation in its acidic region, a process downstream of receptor-linked tyrosine kinases. Vav1 appears to activate Rac1 and Rac2 in phagocytes in response to formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, IgG complexes, lipopolysaccharide (LPS), or Ca^{2+} ionophore, but not to interleukin-8 (IL-8) or leukotriene B_4 (LTB_4) (Kim et al., 2003; Price et al., 2002; Vilhardt et al., 2002; Zhao et al., 2003). Several GAPs are known in these cells, both in the cytosol and in the membrane (Geiszt et al., 2001). By inactivating Rac, these proteins may be involved in termination of the respiratory burst (Szaszi et al., 1999). The inactive form of Rac is kept in a complex with Rho GDP-dissociation inhibitor (RhoGDI), which shields the geranylgeranyl moiety of Rac (Abo et al., 1991; Kwong et al., 1993; Mizuno et al., 1992). The activated, GTP-bound Rac, liberated from RhoGDI, binds to $p67^{phox}$ and associates with its fatty-acid tail into the phospholipids of the plasma membrane (Diekmann et al., 1994). Probably, Rac also interacts with $gp91^{phox}$ itself, to facilitate electron flux through this protein (Diebold & Bokoch, 2001). Another low-molecular-weight GTP-binding protein called Rap1A is associated with flavocytochrome b_{558} in the membrane (Table 52.3) (Quinn et al., 1992). This association is decreased upon protein kinase A-mediated phosphorylation of Rap1A, which suggests that Rap1A may modulate NADPH oxidase activity in response to agents that elevate cAMP in the cells (Bokoch et al., 1991).

FUNCTION OF THE NADPH OXIDASE

It is clear that the functional defects of CGD phagocytes result from the inability of these cells to generate superoxide, hydrogen peroxide, hypochlorous acid, and other products derived from superoxide. However, the bactericidal potency of superoxide and hydrogen peroxide is low, and cells with myeloperoxidase deficiency, which are unable to generate hypochlorous acid, show almost normal bactericidal activity. By contrast, CGD cells incubated with bacteria together with beads covered with glucose oxidase, which generate hydrogen peroxide in the presence of glucose, show clearly improved killing of the bacteria (Johnston & Baehner, 1970). Similar results have been obtained with liposomes filled with glucose oxidase (Gerber et al., 2001). This would indicate that the reactive oxygen compounds themselves are the prime bactericidal agents. An alternative hypothesis has been formulated by the group of Segal (Reeves et al., 2002). These investigators found that mice deficient in leukocyte elastase and cathepsin G have a similar defect in bacterial killing to that of $gp91^{phox}$ knockout mice. They also found that these proteases are liberated from their carbohydrate matrix in the granules by cations, and that this process takes place in neutrophil phagosomes containing ingested bacteria due to an influx of potassium. This influx is induced together with the influx of protons by the accumulation of superoxide, with its negative charge, generated in the phagosomes. In CGD phagocytes, this process does not take place, resulting in decreased protease activity in these organelles and hence decreased bacterial killing. There is now solid evidence that proton translocation from the cytosol to the phagosome through the voltage-gated H^+ channel Hv1 is the most important ion movement to keep the NADPH oxidase activity going (Ramsey et al., 2006, 2009; Sasaki et al., 2006). In addition, evidence from the group of Ligeti indicates that both direct and indirect bactericidal activity of the reactive oxygen species play an important role in the antimicrobial defense of phagocytes (Rada et al., 2004).

Another hallmark of CGD is the occurrence of diffuse granulomas. Some studies have suggested that this too may be caused by the failure of the leukocyte NADPH oxidase in these patients to generate reactive oxygen species, in particular H_2O_2, and a reduced rate of apoptosis (Hampton et al., 2002; Kobayashi et al., 2004). On the other hand, gene expression profiling has shown that CGD neutrophils have constitutively upregulated expression of genes encoding mediators of inflammation and host defense. In contrast, during phagocytosis-induced apoptosis, CGD neutrophils did not display increased Bax expression, as do normal neutrophils (Kobayashi et al., 2004). Thus, granuloma formation in CGD patients may reflect increased proinflammatory activity supporting survival of CGD neutrophils. This aberrant gene expression profile may thus be a direct or indirect effect of diminished reactive oxygen species (ROS) production or ROS-sensitive gene expression. Also, the diminished production of anti-inflammatory mediators by CGD neutrophils and macrophages during phagocytosis of bacteria or apoptotic cells (Brown et al., 2003) may contribute to the persistence of inflammation in CGD. In addition, apoptotic CGD neutrophils also display diminished surface expression of phosphatidyl serine (PS), which may contribute to their delayed removal by macrophages (Hampton et al., 2002; Yamamoto et al., 2002). This last process was shown to involve insufficient priming of the CGD macrophages by IL-4 (Fernandez-Boyanapalli et al., 2009). The cause of the insufficient PS exposure on apoptotic CGD neutrophils remains to be established. The involvement of Th17 and Treg cells in granuloma formation and chronic inflammation is discussed under Animal Models (see below).

An altered B-cell compartment has been suggested based on the reduced number of circulating $CD27^+$ memory B cells in CGD patients (Bleesing et al., 2006). Although we confirmed this, we have not seen a defective antibody response in patients against (viral) infections or vaccinations (Kuijpers & van den Berg, unpublished data). A relationship between the altered B-cell development and the increased risk of autoimmune phenomena in CGD is as yet unproven.

NONPHAGOCYTE NADPH OXIDASES

Several homologs of $gp91^{phox}$ have been identified in cells other than phagocytes. These homologs have conserved the overall structure of six transmembrane domains with four heme-coordinating histidines in the N-terminal part of the molecule, followed by a cytosolic C-terminus that contains highly conserved binding sites for FAD and NADPH. In a new nomenclature, these proteins are now called Nox (NADPH oxidase) proteins, including $gp91^{phox}$ (Nox2). Nox1 (or Mox1) mRNA is expressed

Table 52.3 PROPERTIES OF LOW-MOLECULAR-MASS GTP-BINDING PROTEINS AND RAC REGULATORY PROTEINS IMPLICATED IN ACTIVATION OF THE PHAGOCYTE NADPH OXIDASE

	GTP-BINDING PROTEINS			REGULATORY PROTEINS		
PROPERTY	Rac1	Rac2	RaplA	RhoGD1	P-Rex1	Vav1*
Amino acids	192	192	184	204	1659	877/790
Molecular weight (kDa)						
Predicted	21.4	21.4	20.9	23.4	185	101.4/91.9
By PAGE	22	22	22	26	197	?
PI	8.5	7.6	6.5	4.9	6.2	6.5/6.2
mRNA (kb)	1.1 2.4	1.45	1.6	1.9	6	2.9/2.7
Cellular location†	Cytosol	Cytosol	Membrane	Cytosol	Cytosol	Cytosol
Tissue specificity	Myeloid and other	Myeloid	Widespread	Widespread	Leukocytes and brain	Hematopoietic system
Covalent modification	Isoprenylation	Isoprenylation	Isoprenylation Phosphorylation			Phosphorylation
Homology	92% with Rac2 58% with Rho ~30% with Ras	92% with Rac1 58% with Rho ~30% with Ras	<30% with Rac1/2 <30% with Rho ~50% with Ras	Weak with RasGAP	Tandem PH/DH domain with Rho GEFs 2 DEP and 2 PDZ domains C-terminal -Ins-poly-P 4-phos-phatase	Tandem PH/DH domain with Rho GEFs CH domain Ac region ZF domain 2 SH3 domains 1 SH2 domain Pro-rich region

ABBREVIATIONS: Ac, acidic; CH, calponin homology; DEP, domain found in disheveled, Egl-10, and pleckstrin; DH, diffuse B-cell lymphoma (DBL) homology; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; Ins, inositol; PDZ, Domain found in PSD-95, Dlg, and ZO-1/2; PH, pleckstrin homology; P-Rex1, Pt-dIns(3, 4, 5)P_3-dependent Rac exchanger; Pro, proline; RhoGDI, GDP dissociation inhibitor for Rho proteins; SH, *src* homology; ZF, zinc finger.

*Vav1 has several alternative splice variants.

†Calculated from the amino acid sequences.

‡For resting cells; subcellular localization may vary with activation state of the cell and method of cellular disruption.

in colon, uterus, and vascular smooth muscle cells (Suh et al., 1999). Reactive oxygen species produced by this enzyme seem to be generated intracellularly, in contrast to the situation with the phagocyte NADPH oxidase. Overexpression of Nox1 results in increased cell growth, suggesting a role for these oxygen species in signal transduction for cell growth regulation. Another gp91^{phox} homologue, called Nox4 or Renox, has been detected by in situ RNA hybridization and immunohistochemistry in the renal cortex, predominantly in proximal convoluted tubule epithelial cells (Geiszt et al., 2000; Shiose et al., 2001). It has been suggested that Nox4 may participate in the oxygen-sensing mechanism that regulates the production of erythropoietin. Transfection of Nox4 into 3T3 fibroblasts resulted in enhanced superoxide production; however, these modified cells displayed substantially diminished cell growth. Nox3 is highly expressed in the inner ear, especially in the vestibular and cochlear sensory epithelia, and is crucial for the genesis of otoconia, which are tiny calcium carbonate crystals required for sensing gravity. Nox5 is distinguished from the other family members by its longer N terminus, which contains four EF hand Ca^{2+}-binding motifs. It is found in B and T lymphocytes. Some of these alternative NADPH oxidases, in particular Nox1, Nox3, and Nox4, may interact with specific cytosolic partners for activity regulation and localization—that is, with p22^{phox} and with alternative members of the p47 and p67 protein families (Ambasta et al., 2004; Bánfi et al., 2003; Geiszt et al., 2003; Kawahara et al., 2005; Takeya et al., 2003; Ueno et al., 2005).

Finally, two similar homologs have been discovered that are characterized by an N-terminal extension containing a peroxidase homology domain, a calmodulin-like calcium-binding motif, and an additional transmembrane domain (De Deken et al., 2000). RNA for these proteins, called Duox1 and Duox2, is expressed specifically in the thyroid gland, at the apical membrane of the thyrocytes. Duox2, and perhaps also Duox1, is involved in the iodination of the thyroid hormone, because nonsense mutations in the gene for Duox2 have been identified in patients with congenital hypothyroidism (Moreno et al., 2002).

MOLECULAR BASIS

Deficiency of the phagocyte NADPH oxidase, as seen in CGD, can be the result of any of a number of defects.

First, one of the structural components of the enzyme can be deficient, which is the case when mutations occur in the genes encoding gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, or p67^{phox}. Together, mutations in these five genes account for the overwhelming majority of CGD patients, with defects in the X chromosome-encoded gp91^{phox} found in about 70 percent of the cases and in p47^{phox} in about 20 percent. Defects in p40^{phox}, p22^{phox}, or p67^{phox} are rare. Also very rare are defects in the activation process of the NADPH oxidase. In particular, two patients have been described with a mutation in Rac2, leading to CGD-like symptoms in combination with defects in chemotaxis and in degranulation (Accetta et al., 2011; Ambruso et al., 2000; Williams et al., 2000). This finding indicates that Rac2 is involved not only in the activation of superoxide production but also in the activation of other neutrophil functions. Finally, defects may occur in the process that provides the cell with the substrate for the oxidase (i.e., NADPH). This compound is generated in phagocytes for the largest part in the hexose monophosphate pathway, in the successive G6PD and 6-phosphogluconate dehydrogenase (6PGD) reactions. Occasionally, patients are found with a deficiency of G6PD not only in the erythrocytes but also in the leukocytes (Baehner et al., 1972; Gray et al., 1973; Roos et al., 1999; Van Bruggen et al., 2002). These patients suffer from a mild form of CGD.

The *CYBB* gene for gp91^{phox} has a size of 30 kb, with 13 exons (Table 52.2). It is located on the X chromosome, at Xp21.1. Its promoter region has been clarified in some detail. Eight CGD families are known with mutations in the promoter region of *CYBB* that interfere with binding of the transcription factors Elf-1 and PU.1 (Defendi et al., 2009; Newburger et al., 1994; Roos et al., 2010b; Stasia et al., 2003; Suzuki et al., 1998; Voo & Skalnik, 1999; Weening et al., 2000; Woodman et al., 1995). The transcription of *CYBB* and *NCF1* is enhanced by interferon-γ (IFN-γ) (Cassatella et al., 1990; Weening et al., 1996).

The *CYBA* gene for p22^{phox} has a size of 8.5 kb, with six exons (Table 52.2). It is located on chromosome 16, at 16q24. Its promoter region has been partly characterized (Moreno et al., 2003). The *NCF1* gene for p47^{phox} has a size of 15 kb, with 11 exons, and the *NCF2* gene of p67^{phox} measures 40 kb, with 16 exons (Table 52.2). These genes are located on chromosomes 7 and 1, respectively, at 7q 11.23 and 1q25. Finally, the *NCF4* gene for p40^{phox} has a size of 18 kb, with 10 exons, and is located on chromosome 22, at 22q13.1. Detailed information on the promoter regions of these *NCF* genes has been collected (Li et al., 1997, 1999, 2001, 2002). In the NADPH oxidase genes the exon organizations do not coincide with the various functional regions of the proteins they encode.

The *RAC2* gene is located on chromosome 22, at 22q13. It has a size of 18 kb, with at least seven exons (Table 52.3). The promoter region of this gene has not yet been studied in detail. The Rac2 protein has a molecular weight of 22,000 and consists of 192 amino acids. The *G6PD* gene is located on the X chromosome, at Xq28. It has a size of 18 kb, with 13 exons. The G6PD protein has a molecular weight of 58,000 and consists of 531 amino acids. By binding $NADP^+$, the protein forms catalytically active dimers.

LABORATORY FINDINGS

DIAGNOSIS

Patients suspected of suffering from CGD can be diagnosed by the inability of their blood phagocytes to kill catalase-positive bacteria (e.g., *S. aureus*) in vitro or by their inability to generate reactive oxygen species. The respiratory burst can be measured by oxygen consumption (with an oxygen electrode), by superoxide generation (reduction of ferricytochrome *c* or nitroblue tetrazolium), or by hydrogen peroxide production (oxidation of homovanillic acid or Amplex red). Chemiluminescence with luminol or lucigenin is often used as a very sensitive test of NADPH oxidase activity. Flow-cytometric detection of this activity combines the advantage of sensitivity with the possibility of using nonpurified leukocyte suspensions rapidly derived from whole blood. One such assay, with dihydrorhodamine-1,2,3 (DHR) as the fluorescent detector of hydrogen peroxide production, has proven to be highly reliable and sensitive, even on blood that is 24 to 48 hours old. This assay has therefore replaced superoxide measurements and the NBT slide test as the primary screening assay for CGD in many laboratories (Emmendorffer et al., 1994; van Pelt et al., 1996; Vowells et al., 1996).

CGD SUBGROUP IDENTIFICATION

The NADPH oxidase is composed of multiple subunits, five of which may be affected by mutations leading to CGD (see above, Table 52.2). Subgroup characterization has implications for prognosis and family screening and may someday relate to attempts at gene correction. The methods of subgroup characterization are changing rapidly as molecular techniques become more accessible and less expensive. Therefore, the appropriate choice of technique will vary from region to region and over time. Identification of the protein affected may be desirable before mutation analysis is undertaken. CGD subgroup investigation may therefore start with immunoblot analysis of neutrophil fractions or intracellular staining in permeabilized neutrophils with antibodies against the five NADPH oxidase components. If one of the cytosolic components (p40^{phox}, p47^{phox}, or p67^{phox}) is missing, one can be confident that the gene carrying the mutation has been identified. However, if one of the membrane-bound components (gp91^{phox} or p22^{phox}) is missing, the other is always undetectable as well (Parkos et al., 1989; Verhoeven et al., 1989), because the subunits stabilize each other for full maturation and expression (Porter et al., 1994; Yu et al., 1997). In that case, distinction can usually be made by searching for a mosaic pattern of NADPH oxidase activity or gp91^{phox} expression in the neutrophils of female relatives of the patient, because the gene for gp91^{phox} is located on the X chromosome. Convenient assays for this purpose are the NBT slide test, which scores the NADPH oxidase activity per cell on a semiquantitative basis (Meerhof and Roos, 1986), and the DHR flow-cytometric assay (Roesler et al., 1991; Vowells et al., 1996). Alternatively, one can perform

flow cytometry to detect gp91^{phox} on the neutrophil surface with certain anti-gp91^{phox} antibodies (Mizuno et al., 1988). However, it must be kept in mind that up to one third of all X-linked defects may arise from new mutations in germline cells and will therefore not always be present in the somatic cells from the mother. Thus, failure to define the mother as an X-linked carrier does not disprove the X-linked origin of the disease.

If all subunits of the NADPH oxidase are detectable (the cytochrome subunits and the three other recessive components), even though the activity of this enzyme is absent or strongly decreased, the possibility of so-called ($^+$) variants must be considered (Table 52.4). These are caused by mutations in any of the five subunits that leave the protein expression intact but destroy the enzymatic activity of the assembled oxidase complex. In that case, direct sequencing of all five genes may be entertained. Alternatively, a cell-free oxidase assay may be used to distinguish a defect in a cytosolic component (p40^{phox}, p47^{phox}, or p67^{phox}) from a defect in a membrane-bound component (gp91^{phox} or p22^{phox}). For this assay, neutrophil membranes from the patient are mixed with neutrophil cytosol from a healthy donor (or vice versa), incubated with NADPH, and activated with an amphiphilic agent (low concentrations of sodium dodecyl sulfate [SDS] or arachidonic acid). The resulting oxidase activity can be measured by superoxide formation or oxygen consumption and is used to localize the defect to either the cytosol or the membrane fraction (Curnutte et al., 1987). Identification of the mutated gene that causes the defect in NADPH oxidase activity can also be made if transfection of the patient's Epstein-Barr virus (EBV)-transformed B lymphocytes with retroviral vectors that contain the wild-type cDNA restores this activity (Malech et al., 1997).

Patients with variant forms of X-linked CGD are characterized by a partial loss of gp91^{phox} expression and a proportional loss of NADPH oxidase activity (so-called X$^-$ variants) (Table 52.5). For an exact idea of gp91^{phox} expression, it can be useful to measure the heme content of flavocytochrome b_{558} in the neutrophils (Cross & Curnutte, 1995).

Finally, some patients with CGD-like symptoms are known with defects in either G6PD or Rac2. The first type can easily be recognized by measuring the activity of the G6PD enzyme in the red blood cells and confirming the diagnosis by repeating the assay with a leukocyte preparation (Roos et al., 1999; Van Bruggen et al., 2002). Moreover, one will usually find a mosaic of normally and faintly stained cells in the NBT slide test or DHR assay with purified neutrophils of the mother, because the G6PD gene is localized on the X chromosome. Rac2 deficiency can be recognized by the defect in NADPH oxidase activity in combination with defects in chemotaxis toward complement fragment C5a, IL-8, fMLP, LTB_4, and platelet-activating factor (PAF). The NADPH oxidase activity in PMA-activated neutrophils is normal. A defect in the release of proteins from the azurophilic granules but not from the specific granules or secretory vesicles is also apparent (Ambruso et al., 2000; Roos et al., 1993). The Rac2 deficiency can be diagnosed with certainty only by sequencing the *RAC2* gene.

CARRIER DETECTION

Carrier detection of X-CGD is usually performed by searching for a mosaic pattern of oxidase-positive and -negative neutrophils in the NBT slide test, in the DHR flow-cytometric assay, or in a flow-cytometric assay of gp91^{phox} protein expression. However, because of the random process of X-chromosome inactivation, X-CGD carriers may show a near-normal or a near-pathological pattern in these tests. Once the family-specific mutation is known, it is more reliable to perform carrier detection at the DNA level.

Carriers of autosomal (A) CGD subtypes are difficult to detect. The NADPH oxidase activity and the expression of oxidase proteins are normal in phagocytes from obligate carriers (parents of patients). Hence, detection of carriers of autosomal CGD can be performed only at the DNA level. Unfortunately, for p47^{phox}-deficient CGD, this can be difficult (see Mutation Analysis).

MUTATION ANALYSIS

MUTATION ANALYSIS IN X-CGD

The mutations in more than 1,200 families with X-CGD (OMIM #306400) have been characterized (Cross et al., 2000; Heyworth et al., 2001; Hopkins et al., 1992; Rae et al., 1998; Roos et al., 1996a, 1996b). A summary of all mutations can be found in Roos et al. (2010b) and at http://www.uta.fi/imt/bioinfo/CYBBbase/. The results show that CGD is a very heterogeneous disease, not only clinically and cell biologically but also at the molecular level, even within one subgroup. All possible mutations, except gene inversions, have been found in the *CYBB* gene. These different categories will be briefly mentioned, followed by a more detailed description of a few mutations that have given us insight into the structure–function relation of gp91^{phox}.

Deletions and/or insertions in *CYBB* are encountered in about 35 percent of all X-CGD patients; single nucleotide substitutions are seen in the remaining 65 percent. Table 52.5 provides an overview. One hundred eighty-three mutations (27 percent) were found in more than one unrelated family, with hot spots for a c.252G>A mutation at the end of exon 3 (causing outsplicing of exon 3), several C>T mutations (or its complementary G>A) in CpG sequences (caused by deamination of 5-methylcytosine to thymine, thus creating nonsense mutations from the Arg codon CGA into the TGA stop codon), and an A duplication in a stretch of six A's (causing a frameshift). Four hundred ninety-eight families (39 percent) had unique mutations. Twelve polymorphisms in the *CYBB* gene are known (Heyworth et al., 2001; Roos et al., 2010b; Tarazona-Santos et al., 2008).

Deletions range in size from about 6,000,000 to one single nucleotide. When the deletions are very large, not only the *CYBB* gene but neighboring genes are affected as well. Patients with deletions telomeric from *CYBB* suffer from other clinical syndromes in addition to CGD—for example, Duchenne muscular dystrophy, retinitis pigmentosa, and, as mentioned

Table 52.4 MUTATIONS IN *CYBB* LEADING TO Xb^{+} CGD PHENOTYPE

NO.	NUCLEOTIDE CHANGE	AMINO ACID CHANGE	FUNCTIONAL DOMAIN	FUNCTIONAL ANALYSIS: TRANSLOCATION P47^{phox}/P67^{phox}	FUNCTIONAL ANALYSIS: NADPH BINDING	FUNCTIONAL ANALYSIS: NADPH TO FAD*	FUNCTIONAL ANALYSIS: FAD TO hemes†	REFERENCES
1.	c.160A>G	p.Arg54Gly	2nd TM domain	ND	ND	ND	ND	Roos, 1996
2.	c.161G>T	p.Arg54Met	2nd TM domain	ND	ND	ND	ND	Ishibashi, 2000
3.	c.162G>C	p.Arg54Ser	2nd TM domain	normal	ND	normal	defective	Cross, 1995
4.	c.170C>A	p.Ala57Glu	2nd TM domain	ND	ND	ND	ND	Ariga, 1993
5.	c.890_904del	p.Thr298_Thr302del	?	defective for p67^{phox}/ p40^{phox}	ND	ND	ND	Dusi, 1998
6.	c.907C>T	p.His303Tyr	?	ND	ND	ND	ND	Roos, 2010b
7.	c.907C>A/911C>G	p.His303Asn/Pro304Arg	?	defective	ND	ND	ND	Stasia, 2002; Bionda, 2004
8.	c.1013A>G	p.His338Arg	(binding site for (isoalloxazine ring (of FAD ((338HPFTLTSA345)	ND	ND	ND	ND	Roos, 2010b
9.	c.1022C>A	p.Thr341Lys		normal	ND	ND	ND	Leusen, 2000
10.	c.1105T>C	p.Cys369Arg	loop between FAD and NADPH-binding site	defective	ND	ND	ND	Leusen, 2000
11.	c.1222G>C	p.Gly408Arg	(binding site for (pyrophosphate (of (NADPH	ND	ND	ND	ND	Bakri, 2009
12.	c.1223G>A	p.Gly408Glu		defective	ND	ND	ND	Leusen, 2000
13.	c.1234G>C	p.Gly412Arg		ND	ND	ND	ND	Roos, 2010b
14.	c.1244C>A	p.Pro415His		normal	diminished	ND	ND	Dinauer, 1989; Segal, 1992
15.	c.1244C>T	p.Pro415Leu	((405MLVGAGIGVTPF416)	ND	ND	ND	ND	Stasia, 2008
16.	c.1441A>C	p.Thr481Pro	?	ND	ND	ND	ND	Roos, 2010b
17.	intron11–2A>G	p.Ala488_Glu497del	(alpha (helix ((484D-G504) (over (NADPH- (binding site	normal	ND	defective	ND	Schapiro, 1991; Yu, 1999
18.	intron11–2A>C	p.Ala488_Glu497del?		ND	ND	ND	ND	Roos, 2010b
19.	c.1488_1490delTGA	p.Asp496del		ND	ND	ND	ND	Roos, 2010b
20.	c.1498G>T	p.Asp500Tyr		ND	ND	ND	ND	Kannengiesser, 2008
21.	c.1499A>G	p.Asp500Gly		defective	ND	ND	ND	Leusen, 2000
22.	c.1500T>G	p.Asp500Glu		ND	ND	ND	ND	Kannengiesser, 2008

23.	c.1514T>G	p.Leu505Arg	(binding site for	diminished	ND	diminished	ND	Stasia, 2005
24.	c.1521_1525 delAAAGA/ insCATCTGGG	p.Gln507_Thr509del/ insHisIleTrpAla	(adenine (of NADPH ((504GLKQ507)	normal	ND	ND	ND	Azuma, 1995
25.	c.1609T>C	p.Cys537Arg	binding site for nicotinamide of NADPH (535FLCGPE540)	ND	ND	ND	ND	Rae, 1998
26.	c.1637T>C	p.Leu546Pro	?	ND	ND	ND	ND	Roesler, 1999
27.	c.1702G>A	p.Glu568Lys	?	defective	ND	ND	ND	Leusen, 2000

Adapted from Stasia and Li, 2008.

Abbreviations: ND, not determined: TM, transmembrane. Xb^+, X-linked flavocytochrome b558-positive CGD

*Electron transfer from NADPH to FAD, as measured by iodonitrotetrazolium (INT) reduction in a broken-cell system.

† Electron transfer from FAD to hemes, as measured by INT reduction and cytochrome *c* reduction in a broken-cell system.

Table 52.5 MUTATIONS IN *CYBB* (gp91^{phox})

	NO. OF KINDREDS	FREQUENCY (%)	PHENOTYPE*
Deletions	**281**	**22.2**	**X91^0**
Insertions	**89**	**7.0**	**X91^0**
Splice-site mutations	**247**	**19.5**	**X91^0**
Missense mutations	**246**	**19.4**	**X91^0, X91$^-$, X91$^+$**
Nonsense mutations	**377**	**29.8**	**X91^0**
Promoter mutations	**8**	**0.6**	**X91$^{0\#}$**

*In this nomenclature, the first letter (X) represents the mode of inheritance, while the number (91) indicates the phox component that is genetically affected. The superscript symbols indicate whether the level of protein of the affected component is undetectable (^{0}), diminished ($^-$), or normal ($^+$) as measured by immunoblot analysis.

In neutrophils X91^0 but in eosinophils normal expression and function of gp91^{phox}.

Data collected from 1,267 unrelated kindreds with 1,415 patients (Roos et al., 2010b)

above, McLeod syndrome (a mild hemolytic anemia with decreased levels of Kell erythrocyte antigens due to absence of the K^x protein). When deletions are centromeric from *CYBB*, ornithine transcarbamylase deficiency in addition to CGD can occur (Deardoff et al., 2008). In two families, affected brothers were detected who each carried different deletions in their *CYBB* genes (de Boer et al., 1998). Only in one patient has an in-frame partial exon deletion been reported to lead to expression of the truncated (inactive) gp91^{phox} protein (Schapiro et al., 1991).

Insertions in *CYBB* are usually small in size and, like small deletions, may be caused by slipped mispairing at the DNA replication fork. A few larger tandem duplications of 24, 31, and 40 base pairs have been detected, apparently due to unequal crossing over during meiosis or misalignment during DNA repair (Bu-Ghanim et al., 1995; Rabbani et al., 1993; Roos et al., 2010b). Recently, we have found a much larger duplication of 5.7 kb, ranging from a GT repeat in intron 5 to a similar repeat in intron 8, and the mirror deletion of this *CYBB* fragment in another CGD family (Stasia et al., 2012). Several combinations of small deletions and insertions have been found (Ariga et al., 1995; Heyworth et al., 2001; Rae et al., 1998; Roos et al., 2010b); one of them predicted substitution of three amino acids by four others near the C terminus of gp91^{phox} (Azuma et al., 1995). This mutation led to a stable mutant protein and thus to the X91$^+$ CGD phenotype (Table 52.4). Two large fragments of LINE-1 transposable elements have been identified within *CYBB*. The first one is a 1-kb fragment within intron 5, causing a complicated pattern of misspliced mRNA (Meischl et al., 2000). The second is a 2.1-kb fragment within exon 4, causing a frameshift (Brouha et al., 2002). In both families, the insertion was a recent event because it was not found in the somatic-cell DNA from the patients' mothers. The 2.1-kb fragment was identified as part of an active LINE-1 element on chromosome 2 of the patient's mother. We have also found recently an insertion in *CYBB* derived from a retrotransposed, partially spliced and truncated transcript of *TMF1*, a gene present on chromosome 3. This insertion of 5.8 kb caused inclusion of part of exon 2 of the *TMF1* retrogene in reversed orientation between exon 1 and 2 of the *CYBB* transcript, and predicts premature termination of protein synthesis (De Boer et al., unpublished).

Single nucleotide substitutions at exon–intron boundaries lead to aberrations in mRNA splicing, usually causing skipping of an entire exon. However, this phenomenon was proven in only about half of the cases (in the other half, only genomic DNA was investigated). The percentage of splice-site mutations found in CGD is similar to that found in other diseases (Krawczak et al., 1992). Single nucleotide changes in either exons or introns have sometimes created new splice sites, causing partial exon deletions or insertions, respectively, in the mRNA (de Boer et al., 1992a; Noack et al., 1999, 2001a, Roos et al., 2010b).

Missense and nonsense mutations constitute the most frequently encountered cause of many genetic diseases. Nonsense mutations in *CYBB* affect the level of mRNA for gp91^{phox} to a variable degree but have not been seen in CGD to produce stable truncated proteins. Similarly, deletions, insertions, and splice-site mutations in general also lead to the X91^0 phenotype (no gp91^{phox} protein expression, no NADPH oxidase activity). In contrast, missense mutations do not influence the level of mRNA and may give rise to any of three phenotypes. Single amino acid replacements due to missense mutations can lead to intrinsically unstable gp91^{phox} or to gp91^{phox} unable to be stabilized by interaction with p22^{phox} (X91^0 phenotype), to partial deficiencies in this respect (X91$^-$ phenotype: decreased expression, decreased oxidase activity), or to stable but inactive gp91^{phox} (X91$^+$ phenotype) (Table 52.5). In general, missense mutations in regions of gp91^{phox} not involved in binding of its prosthetic groups (hemes, FAD, NADPH) may weaken the stability of the protein but will often leave some enzymatic activity (X91$^-$), whereas mutations in regions that are involved in this binding will destroy the enzymatic activity, even if the protein is still stable (X91$^+$). Indeed, Kuhns et al. (2010) found that missense mutations in the N-terminus (amino acids 1–309) often leave an appreciable amount of NADPH oxidase activity, with mutations in His222 as an exception (this amino acid is one of the four heme-binding sites). In the C-terminus (amino acids 310–570) of gp91^{phox}, missense mutations in general destroy the superoxide-generating capacity despite leaving the protein expression sometimes nearly or completely intact. This fits with the preponderance of X91$^-$ mutations in the N terminus and of X91$^+$ mutations in the C terminus (Table 52.4, Fig. 52.3) and is explained by the FAD- and NADPH-binding sites in the C terminus of gp91^{phox} (Kuhns et al., 2010; Roos et al., 2010b). Small in-frame deletions and a few splice-site mutations with residual normal mRNA splicing have also been found associated with the X91$^-$ phenotype.

The X91$^+$ mutations can be very informative for our understanding of the properties of the different domains in gp91^{phox}.

Table 52.4 presents an overview. Four X91$^+$ patients have amino acid substitutions in the N-terminal half of gp91^{phox} (Fig. 52.3), one of which (Arg54Ser) provided evidence for the non-identity of the two heme groups in gp91^{phox} (Cross et al., 1995). The other X91$^+$ patients have amino acid substitutions in the C-terminal half of gp91^{phox} (Fig. 52.3). In six of these, the translocation of p47^{phox} and p67^{phox} to cytochrome b_{558} upon neutrophil activation was strongly diminished (Bionda et al., 2005; Dusi et al. 1999; Leusen et al., 1994b, 2000). Apparently, not only the loop over the NADPH cleft is of importance as a docking site for the cytosolic oxidase components, but other regions surrounding this area as well (Fig. 52.5). All together, these results provide strong support for the three-dimensional model of the C-terminal half of gp91^{phox} as constructed by Taylor et al. (1993).

Some X91$^-$ mutations have also proven to be informative (for a complete overview see Roos et al., 2010b, and Stasia & Li, 2008). Tsuda et al. (1998) have described a patient with a His101Tyr mutation, causing 90 percent loss of gp91^{phox} protein expression but total loss of heme incorporation. This indicates that His101 of gp91^{phox} may be involved in heme binding, in accordance with the prediction based on the FRE1 protein (Finegold et al., 1996) and with the need of heme incorporation for maturation and stability of the gp91^{phox}/p22^{phox} complex (Yu et al., 1997). Indeed, site-directed mutation of His101, His115, His209, and His222 in gp91^{phox} has subsequently identified these histidines as the heme ligands and critical for biosynthetic maturation of flavocytochrome b_{558} (Biberstine-Kinkade et al., 2001). A CGD mutation of His338Tyr led to about 80 percent decreased heme levels but almost complete loss of FAD incorporation, in accordance with the His-Pro-Phe-Thr 338–341 motif being involved in FAD binding (Yoshida et al., 1998). This is probably also the explanation for the loss of activity in an X91$^+$ CGD patient with a Thr341Lys mutation in gp91^{phox} (Leusen et al., 2000). Two exceptional X91$^-$ mutations were recently claimed to be macrophage specific—that is, to leave almost normal gp91^{phox} expression and superoxide-generating capacity in circulating neutrophils and monocytes, but to impair these parameters in monocyte-derived macrophages and EBV-transformed B cells (Bustamante et al., 2011). The eight patients, from two separate families, suffered from tuberculous or BCG mycobacterial disease. This appears to be a very limited and informative manifestation of gp91^{phox} deficiency, suggesting a special hypomorphic form of CGD.

Finally, five different single nucleotide substitutions have been identified in the 5′ promoter region of *CYBB* (Defendi et al., 2009; Newburger et al., 1994; Stasia et al., 2003; Suzuki et al., 1998; Weening et al., 2000; Woodman et al., 1995). Mutations at −52 and −53 were found to be located in a binding element for the nuclear transcription factor PU.1 and to suppress the binding of PU.1 and the subsequent promoter activity. Remarkably, these mutations abolished NADPH oxidase activity in the neutrophils, monocytes, and transformed B lymphocytes of the patients, but not in their eosinophils (Defendi et al., 2009; Suzuki et al., 1998; Weening et al. 2000). This finding may explain the mild clinical phenotype of most of these patients (Weening et al., 2000). Apparently, eosinophils have an additional *CYBB* activation mechanism, which has been identified as the transcription factor GATA-1 (Yang et al., 2000).

MUTATION ANALYSIS IN AUTOSOMAL CGD

The number of well-characterized mutations in autosomal CGD is much lower than that in X-CGD, owing to the lower incidence of these forms of the disease. Eighty-seven A22 families, about 350 A47 families, 83 A67 families, and 1 A40 family have been investigated. Table 52.6 presents an overview. The results indicate that the genetic basis of A22 and A67 CGD is as heterogeneous as that of X91 CGD, but A47 CGD is much more homogeneous in origin.

In the 87 A22 CGD families (96 patients) with mutations of p22^{phox} (OMIM #233690), 55 different mutations have been found in the 173 identified alleles (for a detailed overview see Roos et al., 2010a, and http://www.uta.fi/imt/bio info/CYBAbase/). In 66 families, the patients carried homozygous deficiencies; in 21 families, the patients were compound heterozygotes. Twelve mutations (22 percent) were found in more than 1 unrelated family, with hot spots

Table 52.6 **MUTATIONS IN *CYBA, NCF1*, AND *NCF2***

	CYBA* (P22^{phox})		***NCF1***† (P47^{phox})		***NCF2***‡ (P67^{phox})	
	NO. OF ALLELES	**FREQUENCY**	**NO. OF ALLELES**	**FREQUENCY**	**NO. OF ALLELES**	**FREQUENCY**
Deletions	42	24.3%	7	11.1%	48	28.1%
Insertions	17	9.8%	1#	1.6%	8	4.7%
Splice-site mutations	29	16.8%	11	17.5%	38	22.2%
Missense mutations	65	37.5%	6	9.5%	41	24.0%
Nonsense mutations	20	11.6%	38	60.3%	36	21.0%

*Mutations in *CYBA*: 87 families with 173 identified alleles in the 96 patients

†Mutations in *NCF1* (other than GT deletion at the start of exon 2): 42 families with 63 identified alleles in the 53 patients

‡Mutations in *NCF2*: 83 families with 171 identified mutations in the 95 patients (one allele carries two mutations)

#Deletion/insertion

of missense mutations at c.70C>A (p.Gly24Arg) in 9 families (14 alleles) and c.268C>T (p.Arg90Trp) in 8 families (14 alleles). Forty-three families (49 percent) had unique mutations. Twelve polymorphisms are known in the $p22^{phox}$ protein (Bedard et al., 2009; Cross et al., 2000; Roos et al., 2010a). A single $A22^{+}$ patient has been found with a Pro156Glu substitution (Dinauer et al., 1991). Pro156 is in a proline-rich region of $p22^{phox}$ that serves as a binding region for an SH3 domain in $p47^{phox}$. Apparently, the Pro156Glu substitution destroys the interaction between these proteins and hence the activation of the NADPH oxidase (Leto et al., 1994; Leusen et al., 1994a; Sumimoto et al., 1994). This substitution is the first recognized mutation that disturbs an SH3–proline interaction and leads to a genetic disease.

The mutations that cause A47 CGD have long puzzled investigators. In more than 300 unrelated patients with $p47^{phox}$ deficiency (OMIM #233700), a dinucleotide deletion has been found at a GTGT tandem repeat corresponding to the first four bases of exon 2 in the *NCF1* gene (Casimir et al., 1991; Iwata et al., 1994; Noack et al., 2001b; Roesler et al., 2000; Roos et al., 1996a, 2010a; Vazquez et al., 2001; Volpp and Lin, 1993). In all but 42 of these families (53 patients or about 15 percent), the GT deletion (ΔGT) appeared to be homozygous (Roos et al., 2007, 2010a). In these 42 exceptions, 20 families had patients who were heterozygotes for the GT deletion and one additional mutation, and 22 families had patients with mutations other than ΔGT on both alleles of *NCF1*. Of these last 22 families, 20 had patients who were homozygotes for a non-ΔGT mutation and 2 families were non-ΔGT compound heterozygotes. Three mutations (13 percent) were found in more than one unrelated family, with a hot spot for the nonsense mutation c.579G>A, which changes the TGG codon for Trp193 into the TGA stop codon. Nineteen of the nonhomozygous ΔGT families (45 percent) had unique mutations. For an overview of these mutations see Roos et al. (2010a) and http://www.uta.fi/imt/bioinfo/NCF1base/. The spectrum of these non-ΔGT mutations is as diverse as that in the other CGD genes (Table 52.6).

Obviously, the ΔGT-bearing allele of *NCF1* is the most common CGD-causing allele in the population, carried by approximately 1 in 500 individuals. The reason for this predominance is that most normal individuals have on each chromosome 7 two $p47^{phox}$ pseudo-genes, which colocalize (one on each side; Antonell et al., 2006) with the functional *NCF1* gene to 7q11.23. These pseudogenes are more than 99 percent homologous to *NCF1* but carry the GT deletion, which renders them inactive because the predicted protein from these pseudogenes contains a premature stop codon. Recombination events between *NCF1* and the pseudogenes lead to the incorporation of ΔGT into *NCF1*, and thus to CGD (Goerlach et al., 1997; Roesler et al., 2000; Vazquez et al., 2001). To date, nine polymorphisms have been recognized in *NCF1* (Cross et al., 2000; Roos et al., 2010a), but the identification of these variations is complicated by the presence of the pseudogenes. Unfortunately, this complicated genetic background of A47 CGD renders reliable carrier testing difficult, because normal individuals appear to be "heterozygous" for the GT deletion by virtue of possessing the pseudogenes. Allele-specific PCR amplification can be used to solve this problem but is not easily applicable (Noack et al., 2001b). A gene-scan method has been developed for determining the relative number of *NCF1* and pseudogenes in genomic DNA. With this test, patients and carriers with the GT deletion can be distinguished from normal individuals and from each other (Dekker et al., 2001). However, it is not yet clear whether a compound protein with the GTGT sequence from *NCF1* followed by the 3′ sequence of the pseudogenes is expressed and has oxidase-promoting activity (Heyworth et al., 2002). We have found indications that the mRNA for such fusion genes is not processed correctly and therefore is unstable (unpublished). In all A47 CGD patients analyzed thus far, the mRNA levels for $p47^{phox}$ were normal, but the amount of $p47^{phox}$ protein in each was undetectable. Thus, only $A47^{0}$ patients have been identified to date.

In the 83 characterized A67 CGD families with abnormal $p67^{phox}$ (OMIM #233710), the 95 patients carry 54 different (and 3 unidentified) mutations among the 171 total alleles affected (one mutation in 8 families is always found in combination with another mutation on the same allele) (Table 52.6) (for a detailed review see Roos et al., 2010a, and http://www.uta.fi/imt/bioinfo/NCF2base/). In 70 families, the patients are homozygous for one mutation; in 13 they are compound heterozygotes. Eighteen mutations (33 percent) were found in more than one unrelated family, with hot spots of G>A splice-site mutation at the start of intron 4 in eight families and a c.304C>T nonsense mutation (p.Arg102X) in six families. Thirty-six families (43 percent) had unique mutations. Twelve polymorphisms in *NCF2* are known (including the 5′ untranslated region), six of which predict amino acid substitutions. In some of the A67 CGD patients, the level of mRNA for $p67^{phox}$ is normal, but in most the $p67^{phox}$ protein is undetectable. However, in four families with three different mutations about half the normal amount of protein was found. Two missense mutations (p.Asp108Val and p.Ala202Val) lead to concomitant decrease in NADPH oxidase activity, presumably due to structural changes in $p67^{phox}$ that preclude efficient oxidase activation (Köker et al., 2009; Yu et al., 2008). In the last family, the mutation predicts an in-frame deletion of Lys58 from the protein and apparently results in the expression of a nonfunctional $p67^{phox}$ that fails to translocate to the plasma membrane because of the inability of this protein to bind Rac (Leusen et al., 1996).

Recently, one patient has been described with $p40^{phox}$ deficiency (Matute et al., 2009). This patient suffered from granulomatous colitis, perianal rash, perioral eczema, sinusitis oral ulcers, and an undefined perioperative infectious episode, but no other symptoms frequently seen in CGD patients. His neutrophils produced normal amounts of superoxide after stimulation with PMA or fMLP, but strongly reduced amounts after phagocytosis of serum-treated zymosan, IgG-coated beads or serum-opsonized *S. aureus*. Killing of opsonized *S. aureus* by the patient's neutrophils was significantly impaired. The $p40^{phox}$ expression by the cells from the patient and his father was about half normal. The patient proved to be a compound heterozygote for two mutations in *NCF4*. On the paternal allele he had a 10-base pair duplication that predicts

a frameshift and a premature stop codon, and on the maternal allele a nucleotide substitution that predicts a missense Arg105Gln mutation in the PX domain. The first mutation destroyed the $p40^{phox}$ protein expression ($A40^0$), whereas the second mutation led to normal expression of a nonfunctional protein ($A40^+$).

MUTATION ANALYSIS IN RAC2 DEFICIENCY

Rac2 deficiency has been described in two patients only (OMIM #602049) (Accetta et al., 2011; Ambruso et al., 2000; Williams et al., 2000), although the cellular deficiencies exactly match those described in another patient with neutrophil defects of unknown etiology (Roos et al., 1993). The first patient with proven Rac2 deficiency suffered from severe bacterial infections and poor wound healing, symptoms that, together with late separation of the umbilical stump, leukocytosis, and absence of pus in infected areas, also observed in the patient, are reminiscent of leukocyte adhesion deficiency (LAD). Indeed, neutrophil studies indicated a defect in chemotaxis toward C5a, fMLP, and IL-8, but in contrast to LAD-I, normal expression of β_2 integrins (CD 18) on the leukocytes (see Chapter 53). Moreover, despite a normal content of enzymes of the azurophil granules, strongly decreased release of these enzymes after neutrophil activation was found, but the release of lactoferrin from the specific granules was normal. Neutrophil polarization and actin polymerization in response to fMLP were also deficient, as were transient adhesion to the L-selectin ligand GlyCAM-1 and stable adhesion to fibronectin. Neutrophils from this patient failed to respond with superoxide production after activation with fMLP or C5a, but responded normally with PMA. Thus, this syndrome combined defects seen in LAD with those observed in CGD and in addition included defects in degranulation and actin-dependent functions, as well as abnormal T-cell development resulting in lymphopenia and absence of T-cell–receptor excision circles on newborn screening (Accetta et al., 2011). Mutation analysis of the Rac2 gene showed a single heterozygous c.169G>A transition in both patients, predicting a substitution of Asp57 for Asn. This Asp57 is in a conserved D-X-X-G motif involved in GTP binding to Ras GTP-ases. Recombinant $Rac2^{D57N}$ bound GDP normally but failed to bind GTP (Williams et al., 2000).

The involvement of Rac2 in multiple neutrophil functions had already been deduced from studies with Rac2 knockout mice (Roberts et al., 1999). However, in these studies it was shown that both Rac2 alleles had to be disrupted for defects to become apparent. The mechanism of the dominant negative effect of $Rac2^{D57N}$ in the patients was clarified by Gu et al. (2001), who found that $Rac2^{D57N}$ binds the guanine nucleotide exchange factor (GEF) TrioN normally, but this binding has no effect on the exchange of GDP for GTP, in contrast to the increased exchange seen with wild-type Rac2. These results suggest that $Rac2^{D57N}$ sequesters GEFs in neutrophils by binding these factors but, because of the impaired binding of GTP, do not release these factors, resulting in decreased turnover rates of Rac2 and possibly other Rho-like GTPases that use these GEFs.

MUTATION ANALYSIS IN G6PD DEFICIENCY

Deficiency of G6PD is one of the most common genetic defects in humans (OMIM #305900), probably because of the protection of G6PD-deficient erythrocytes against proliferation of malaria parasites. The clinical expression of G6PD deficiency varies from mild hemolytic anemia induced by infections or drugs (World Health Organization [WHO] class 3) to chronic nonspherocytic hemolytic anemia with attacks of severe anemia induced by infections and drugs (WHO class 1). The clinical symptoms are often restricted to the erythrocytes, for several reasons. One is that G6PD and the next enzyme in the hexose monophosphate pathway (6PGD) catalyze the only NADPH-generating reactions in cells that lack mitochondria (i.e., erythrocytes). These cells need NADPH for protection against oxidative stress. Other reasons for G6PD deficiency effects in erythrocytes are the long survival time of these cells in the circulation and their lack of protein synthesis. G6PD mutant enzymes often have decreased protein stability, causing a decline in enzyme activity, especially in long-living cells that cannot generate new proteins. A total lack of G6PD activity is probably incompatible with life, because mutations in G6PD concern missense mutations or small in-frame deletions, but not frameshift mutations, gross deletions, or nonsense mutations (with one exception near the C-terminus of the protein) (Beutler et al., 1996).

Occasionally, patients with G6PD deficiency have been described with a decreased neutrophil respiratory burst after activation with opsonized particles, as well as an increased susceptibility of patients to bacterial or fungal infections (Baehner et al., 1972; Cooper et al., 1972; Gray et al., 1973; Mamlok et al., 1987; Roos et al., 1999; Van Bruggen et al., 2002; Vives Corrons et al., 1982). In general, the G6PD activity in these cells needs to be below 5 percent of normal for such impairment to occur (Baehner et al., 1972). This situation may arise when mutations in G6PD severely decrease the enzyme activity even in newly synthesized protein. To date, information about mutations that give rise to these CGD-like symptoms is restricted to a few families. In one of these, we found replacement of Pro172 by Ser, leading to a thermolabile variant G6PD with about 15 percent of normal activity (Roos et al., 1999). In two other families with mild CGD and G6PD deficiency we found a triplet nucleotide deletion that predicted deletion of Leu61. G6PD protein expression and enzyme activity in all blood cells were extremely low (Van Bruggen et al., 2002).

PHENOTYPE–GENOTYPE CORRELATION

In general, the clinical symptoms in A22 and A67 subgroups of CGD appear to be as severe as in $X91^0$ CGD. Clinical comparisons between X-CGD and A47 CGD patients have been made by several investigators, with the general contention that X-CGD patients follow a more severe clinical course than that of A47 CGD patients (Gallin et al., 1991; Jones et al., 2008; Margolis et al., 1990; Martire et al., 2008; Van den Berg

et al., 2009; Weening et al., 1985a; Winkelstein et al., 2000). The milder disease course is probably due to residual superoxide or hydrogen peroxide formation by $p47^{phox}$-deficient neutrophils (Bemiller et al., 1991; Cross et al., 1994; Cross & Curnutte, 1995; Vowells et al., 1996). In a recent paper, Kuhns et al. (2010) show that not the residual $gp91^{phox}$ protein expression but the residual NADPH oxidase activity determines the chance of survival of X-CGD patients. Analysis of the correlation between the clinical course and residual NADPH oxidase activity has not been performed yet.

It might be expected that patients with the $X91^-$ phenotype, with 3 to 30 percent of residual NADPH oxidase activity in their phagocytes, would follow a more benign clinical course than that of patients with the $X91^0$ or $X91^+$ phenotype. This generally appears to be the case, but it is not universally true (Roos et al., 1992). Female carriers of X-CGD mutations may occasionally present with clinical manifestations of the disease similar to those of hemizygous patients. In general, this concerns only carriers with less than 10 percent of normal phagocytes (Roos et al., 1996a; Wolach et al., 2005), but healthy carriers with less than 10 percent of normal phagocytes are also known (Roos et al., 1986).

Functional polymorphisms in the oxygen-independent antimicrobial systems or other host defense elements play an important role in this respect. Foster et al. (1998) found that in a cohort of 129 clinically well-defined CGD patients (104 X-linked, 25 autosomal recessive), an increased risk for abnormal granulomatous or inflammatory processes leading to gastrointestinal or urogenital complications was strongly associated with a polymorphism in the promoter region of myeloperoxidase that increases transcriptional activity, and with the NA2 allele of the Fcγ receptor IIIb, which reacts less efficiently with IgG1-opsonized microorganisms than does the NA1 allotype. Autoimmune and rheumatologic disorders were seen more frequently in CGD patients with variant alleles of mannose-binding lectin and Fcγ receptor IIa.

The question as to how oxidative and nonoxidative mechanisms cooperate in intraphagosomal killing has been addressed by Reeves et al. (2002), who found that charge compensation for superoxide generation is mediated to some extent by potassium influx. These potassium ions are instrumental in liberating proteases from the proteoglycan matrix in the azurophil granules that have fused with the phagosome, but also cause a temporary increase in intraphagosomal pH, needed for optimal functioning of these proteases. While some of these antimicrobial systems may operate independently, the combination of their activities is synergistic in the successful containment of the invading pathogens. Such gene modifiers may explain the variability in the infectious burden in CGD. Also, the prevalence of specific pathogens in certain patients, such as *Nocardia* or atypical mycobacterial strains, may depend on these nonoxidative systems (Dorman et al., 2002; Weening et al., 2000; Winkelstein et al., 2000). In addition, weak nonoxidative defense systems may also allow catalase-negative microorganisms to infect CGD patients. Finally, this variability of nonoxidative defense systems may explain the beneficial effects of rhIFN-γ in vivo in CGD patients (see below), despite its lack of effect on NADPH oxidase activity: many of the nonoxidative systems are activated and modulated by microbial products and a series of temporally expressed cytokines such as IFN-γ.

PRENATAL DIAGNOSIS

Prenatal diagnosis of CGD can be performed by analysis of the NADPH oxidase activity of fetal blood neutrophils (Newburger et al., 1979), but fetal blood sampling cannot be undertaken before 16 to 18 weeks of gestation. Instead, analysis of DNA from amniotic fluid cells or chorionic villi now provides an earlier and more reliable diagnosis for families at risk. In cases where the family-specific mutation is known, this analysis can be performed by PCR amplification and sequencing of the relevant genomic DNA area. Between our laboratories and that at Scripps, we have analyzed more than 80 pregnancies at risk for X-CGD (de Boer et al., 1992b). The same strategy can be used for prenatal diagnosis of other CGD subtypes, although this may be more complicated if the parents carry different mutations. We have performed prenatal diagnosis in two families with a $p67^{phox}$-deficient CGD patient and in four families with $p47^{phox}$-deficient CGD. In the latter four families, the deficiency proved to be due to mutations other than the GT deletion at the start of exon 2 in *NCF1*, which was proven by sequencing of *NCF1*-specific PCR products (de Boer et al., 2002).

If the family-specific mutations are not known, different methods must be applied. Partial or complete gene deletions can be recognized by restriction fragment length polymorphism (RFLP) on Southern blots or by multiplex ligation-dependent probe amplification (MLPA) analysis of genomic DNA, but more subtle abnormalities require the use of allele-specific markers. Sometimes it is possible to identify the family-specific mutation quickly in the genomic DNA of the mother (in the heterozygous state) before analysis of the fetal DNA.

ANIMAL MODELS, COMPARISON WITH HUMAN DISEASE

Natural animal models of CGD are not known. However, Dinauer's group has succeeded in constructing a mouse model of X-linked CGD through *Cybb* gene targeting of murine embryonic stem cells (Pollock et al., 1995). With a similar technique, $p47^{phox}$ and $p40^{phox}$ knockout mice have been constructed (Ellson et al., 2006; Jackson et al., 1995; Reeves et al., 2002). Such models are of great value for testing the safety and efficacy of correction of the genetic defect by gene transfer technologies (see Future Directions, below). In addition, the clinical differences between the various subgroups of CGD and new therapeutic strategies can be studied in detail with these models. By comparison with elastase knockout mice, cathepsin-G knockout mice, and elastase/cathepsin-G double-knockout mice, the importance of the oxidative and nonoxidative microbicidal mechanisms was studied in vivo (Tkalcevic et al., 2000). $P40^{phox}$ knockout

mice have been used to study the exact function of this protein (Ellson et al., 2006). Mice deficient in NADPH oxidase components have also been used to study the involvement of this enzyme system in oxygen sensing, blood pressure regulation, and pathogenesis of atherosclerosis (Archer et al., 1999; Brandes et al., 2002; Fu et al., 2000; Hsich et al., 2000; Kirk et al., 2000; O'Kelly et al., 2000; Wang et al., 2001). Finally, $p22^{phox}$-deficient mice have recently been discovered (Nakano et al., 2008). The mouse strain nmf333, with a balance disorder like *Nox3*-mutated mice, turned out to have a mutation in the *Cyba* gene that destroyed $p22^{phox}$ protein expression and NADPH oxidase activity. However, a role for one or more of the classical phox proteins in human vascular disease or gravity sensing cannot be deduced from the clinical symptoms of CGD patients thus far.

Recently, a role for indoleamine 2,3-dioxygenase (IDO) was suggested to be important in CGD pathology. IDO suppresses T-cell responses (hence promoting tolerance), tumor resistance, chronic infection, autoimmunity, and allergic inflammation. During inflammation, IDO is upregulated in dendritic cells and phagocytes by proinflammatory stimuli—most notably IFN-γ. The IDO enzyme supposedly uses superoxide as a "cofactor" for oxidative cleavage of the indole ring of tryptophan, yielding an intermediate that deformylates to L-kynurenine.

Patients with CGD suffer not only from infectious disease but also from chronic inflammatory conditions, most prominently granuloma formation—even when the pathogen has most likely been already eliminated. The reason for the exaggerated inflammatory response has remained unclear. Recent studies in CGD $p47^{phox}$ knockout mice have demonstrated that a superoxide-dependent step in tryptophan metabolism along the kynurenine pathway is blocked, leading to unrestrained γδ T-cell reactivity, dominant production of IL-17, defective regulatory T-cell ($Foxp3^+$ Treg) activity, and acute lethal inflammation upon pulmonary *Aspergillus* infection. Complete cure and reversal of the hyperinflammatory phenotype upon lung infection were achieved by replacement therapy with a natural kynurenine distal to the blockade in the pathway, combined with the co-administration of rIFN-γ or depletion of IL-17. This treatment restored production of downstream immunoactive metabolites and enabled the emergence of regulatory T cells, such as the $Foxp3^+$ Treg cells (Romani et al., 2008). IDO seems to be blocked posttranslationally in CGD mice, because IFN-γ could still induce IDO expression in $p47^{phox}$ knockout PMN, but these cells were unable to mediate tryptophan conversion to L-kynurenine, 3-hydroxykynurenine, and quinolinate. When this idea was put to the test in CGD patients, remarkable differences with the mouse model came to light. CGD monocytes, dendritic cells, and neutrophils all generated normal amounts of kynurenine when stimulated by rIFN-γ and LPS. Moreover, urinary and serum levels of kynurenine and other tryptophan metabolites were elevated rather than suppressed in CGD patients (De Ravin et al., 2010; Jürgens et al., 2010). Also, in the human system, cytochrome b_5 together with cytochrome P450 and NADPH, rather than superoxide, acts as the IDO-activating system (Maghzal et al., 2008). Thus, it appears that the excessive inflammatory reactions in CGD patients, the formation and maintenance of granulomas, and the susceptibility of these patients to develop autoimmune diseases are independent of IDO activity but may still be dependent on Th17 and/or Treg. Indeed, as was recently shown, ROS production by macrophages is instrumental in the induction of Tregs that suppress the activation and proliferation of effector T cells. This was observed both with CGD monocyte-derived macrophages as well as with $p47^{phox}$-mutated rat macrophages in vitro and in vivo (Kraaij et al., 2010).

TREATMENT OPTIONS

The prognosis for patients with CGD has dramatically improved since the disorder was first described in the 1950s as fatal granulomatous disease. The grim prognosis in these earlier times was borne out in retrospective epidemiological studies. In one such review of 31 patients followed between 1964 and 1989, actuarial analysis showed 50 percent survival through the third decade of life (Finn et al., 1990). In another retrospective study of 48 patients followed between 1969 and 1985 in Paris, the survival rate was 50 percent at 10 years of age, with substantially fewer deaths thereafter (Mouy et al., 1989).

Several large series have been more recently published. Winkelstein et al. reported a retrospective study on 368 CGD patients in the United States (Winkelstein et al., 2000), followed by an Italian registry on 60 patients (Martire et al., 2008), the UK and Ireland CGD registry incorporating 94 patients (Jones et al., 2008), and a study representing larger Europe (not including Italy, Ireland, and the UK) on 429 patients early in 2009 (Van den Berg et al., 2009). In contrast to the mortality rate of 17.5 percent in the U.S. study, the survival curve of the Italian patients indicated a relatively linear death rate between the ages of 1 and 15 years and then a sudden fall in the third decade. The survival rate was 46 percent at 25 years, and this was maintained over the next 10 years. Compared to the earlier reports, CGD patients showed a better outcome, considering that one third of the deaths were due to complications after bone marrow transplantation (Martire et al., 2008). More than 75 percent of the patients received cotrimoxazole and itraconazole prophylaxis, but still half of the deaths were ascribed to *Aspergillus* spp. infections (Martire et al., 2008). In the UK and Ireland almost all CGD patients received antibiotic and antifungal prophylaxis. Despite prophylaxis, estimated survival was still only 55 percent at age 30 years. In the larger European study the median life expectancy was relatively high (37.8 years for X-linked patients, 49.6 years for autosomal recessive patients), even though only 71 percent of the patients received antibiotic maintenance therapy and 53 percent additional antifungal prophylaxis (van den Berg et al., 2009). Kuhns et al. (2010) found that the critical predictor of survival was residual superoxide production. Those with the highest residual superoxide production, regardless of genotype, had survival rates over 80 percent by age 40, while those with the lowest superoxide production had survival rates around 50 percent by about age 30. Interestingly, mortality rates did not diverge between those two extremes of

superoxide production until after age 20, suggesting that mortality may correlate with accumulated end-organ toxicities, such as portal hypertension (Feld et al., 2008; Hussain et al., 2007). Although CGD will always remain a clinical challenge, better figures on survival are to be expected for the future with the newer antifungal drugs now available (see below).

One important factor in the improving prognosis for CGD patients is the emergence of several centers in the world that have concentrated on the study and care of scores of patients with CGD (e.g., the National Institutes of Health [Bethesda, MD], Scripps Clinic/Stanford University [California], and the Academic [Pediatric] Clinics in Amsterdam, London, Munich, Paris, and Zürich). Many insights have been gained from the coordinated treatment of these large groups of patients, and this information has been widely disseminated through the literature and through personal communications.

The cornerstones of treatment of CGD are as follows: (1) prevention of infections through immunizations and avoidance of certain sources of pathogens; (2) use of prophylactic trimethoprim-sulfamethoxazole or dicloxacillin; (3) use of prophylactic antifungal medications such as itraconazole; (4) use of prophylactic recombinant human interferon-γ (rhIFN-γ); (5) early and aggressive use of parenteral antibiotics; and (6) surgical drainage or resection of recalcitrant infections. Of these six components, the most important are prophylactic antimicrobials and early intervention in infections before they have the potential to overwhelm the compromised immune system of the CGD patient.

There are multiple ways in which CGD patients can minimize their risk of infection. They should receive all routine immunizations (including live-virus vaccines) on schedule as well as influenza vaccine yearly. Lacerations and skin abrasions should be promptly washed with soap and water and rinsed with an antiseptic agent such as 2 percent hydrogen peroxide or a Betadine solution. The risk of developing perirectal abscesses can be lessened by careful attention to hygiene, avoidance of constipation, and frequent soaking in warm, soapy baths. The frequency of pulmonary infections can be reduced by refraining from smoking, not using bedside humidifiers, and avoiding sources of *Aspergillus* spores (e.g., hay, straw, mulch, decaying plant material, rotting wood/sawdust, and compost piles). Professional dental cleaning, flossing, and antibacterial mouthwashes can help prevent gingivitis and periodontitis.

Chronic prophylaxis with trimethoprim-sulfamethoxazole (5 mg/kg per day of trimethoprim given orally in one or two doses up to a maximum dose of 160 mg trimethoprim per day) decreases the number of bacterial infections in CGD patients—by more than half in series of 48 and 36 patients, respectively (Margolis et al., 1990; Mouy et al., 1990). In sulfa-allergic patients, dicloxacillin (25–50 mg/kg per day) or trimethoprim alone (100 mg daily) can be used, although there is less documentation of the efficacy of these antibiotics in the prophylactic setting. At one point, there was concern that the sustained use of prophylactic antibiotics could lead to an increased risk of fungal infections. Fortunately, the data do not bear out this concern (Margolis et al., 1990).

The effectiveness of antifungal prophylaxis is far less clear. Itraconazole, a newer orally active triazole antifungal antibiotic, has been reported to be an effective prophylactic agent in one study (Mouy et al., 1994) and had significant activity in a randomized prospective trial (Gallin et al., 2003). However, fungal infections still occurred in patients receiving antifungal prophylaxis even when documented itraconazole blood levels were within the therapeutic range, albeit at markedly reduced rates compared to those not on prophylaxis (Gallin et al., 2003; Jones et al., 2008; Martire et al., 2008). *Aspergillus* spp. stand at the top, warranting an even more aggressive approach to this infection in CGD patients. Most clinical data are from a time when diagnostic tools such as high-resolution CT scans and MRI were not available. Also, many successful antifungals currently in use, such as voriconazole or posaconazole, had at the time not yet been developed. Better diagnosis and treatment should lead to a reduction in *Aspergillus*-related mortality in the future. However, the long-term safety of these newer antifungal drugs has not been fully investigated in the CGD setting.

The efficacy of rIFN-γ as a prophylactic agent in CGD was evaluated in a phase III multicenter, double-blind, randomized, placebo-controlled study involving 128 patients. The results, published in 1991, showed a 70 percent reduction in the risk of developing a serious infection in the rIFN-γ–treated group compared to placebo (Gallin et al., 1991). This benefit is maintained in patients treated for longer periods of time, as reported in two phase IV studies (Bemiller et al., 1995; Weening et al., 1995) and a summary of all published trials with rIFN-γ (Marciano et al., 2004b). In the more recent European cohort studies—being both retrospective and partially prospective—rIFN-γ was used in the treatment of active infection without much evidence of efficacy (Jones et al., 2008). In one study long-term prophylaxis with rIFN-γ did not significantly change the rate of total infections per patient-year compared to controls receiving antimicrobial prophylaxis alone (Martire et al., 2008).

Side effects of rIFN-γ are generally minimal, even with prolonged therapy, and are mainly restricted to mild headaches and low-grade fevers within a few hours after administration. The recommended dose is 0.05 mg/m^2 given subcutaneously three times per week. (For infants weighing <0.5 m^2, the recommended dose is 0.0015 mg/kg given subcutaneously three times per week.) The significant clinical improvements in the phase III patients were not accompanied by improvements in superoxide production, a finding confirmed in subsequent studies (Davis et al., 1995; Mühlebach et al., 1992; Woodman et al., 1992). It now appears that rIFN-γ augments host defense in the vast majority of CGD patients, regardless of genetic subtype, by means other than reversing the respiratory burst defect. The effect of rIFN-γ on changing the enzymatic IDO levels, thereby generating L-kynurenine metabolites in dendritic cells and macrophages of (inbred) CGD knockout mice (Romani et al., 2008), does not seem to be operative in human CGD patients (De Ravin et al., 2010; Jürgens et al., 2010).

One of the most serious errors in the management of CGD patients is the failure to treat potentially serious infections promptly with appropriate parenteral antibiotics and to continue therapy long enough to eradicate them. Once bacterial and fungal infections become well established and gain

the upper hand in a CGD patient, even the best-chosen antibiotics may be relatively ineffective. Therefore, early intervention is of critical importance. It is usually necessary to begin antibiotic therapy empirically before culture results are available; in these cases, the antibiotics should be chosen to provide strong coverage for *S. aureus* and gram-negative bacteria, including *B. cepacia* complex (e.g., a combination of nafcillin and ceftazidime or a carbapenem; aminoglycosides are typically ineffective against *Burkholderia*). If the infection fails to respond within 24 to 48 hours, then more aggressive diagnostic procedures should be instituted to identify the responsible microorganism. Additional empirical changes in antibiotic coverage may be warranted, such as adding high-dose intravenous trimethoprim-sulfamethoxazole to cover ceftazidime-resistant *B. cepacia* and *Nocardia*.

If fungus is identified or strongly suspected, antifungal therapy should be started even before the diagnosis is confirmed. *Aspergillus* infections of the lungs and bones are the most common fungal infections and often require prolonged treatment. The choice of antifungal is still evolving and depends on the center, the availability of different agents, and patient tolerance. In general, the newer triazole antifungals are better tolerated and have better activity than amphotericin derivitives against most molds other than the *Mucorales*. The non-f*umigatus Aspergillus* infections as well as dematiaceous molds in CGD are typically amphotericin resistant. In addition, CGD patients infrequently develop infections due to *Mucorales* species that would require amphotericin (Vinh et al., 2009a). When a lipid formulation of amphotericin B is used, treatment should be continued longer than typically deemed necessary, and based on radiographic, clinical, and culture data and the return of the erythrocyte sedimentation rate to its "normal" baseline for the patient or of the C-reactive protein to normal. Most patients are assumed to require prophylactic treatment. New triazole antifungals for intravenous and oral use, itraconazole, voriconazole, and posaconazole, have already shown great benefit in patients with refractory fungal infections and are the preferred agents in some centers for the empiric treatment of all filamentous mold infections, including in CGD patients. The echinocandin antifungals (caspofungin, micafungin, anidulafungin) can be used for the treatment of refractory *Aspergillus* infections in patients unresponsive to or unable to receive azoles or lipid-formulated amphotericin B. Certain fungal infections may progress under appropriate therapy in CGD, especially with some of the newly characterized fungi that are morphologically similar to *A. fumigatus* but have distinct and aggressive behaviors in CGD patients (Verweij et al., 2008; Vinh et al., 2009b, 2009c). Early treatment with additional intravenous antifungals and surgical resection of infected tissues must be given (Jabado et al., 1998; Segal et al., 1998; Van't Hek et al., 1998; Walsh et al., 2002).

Surgery plays an important role in the management of CGD patients. As noted above, surgical drainage (and sometimes excision) of infected lymph nodes and abscesses involving the liver, skin, rectum, kidney, and brain is often necessary for healing, particularly for the visceral abscesses. In cases of highly aggressive fungal or bacterial infections that fail to respond to the medical and surgical approaches outlined above, granulocyte transfusions may be helpful.

Special caution must be exercised in CGD patients regarding transfusions, whether they be granulocytes, erythrocytes, or platelets. Some CGD patients have McLeod syndrome, as discussed earlier. Since the K^x protein is absent and other Kell antigens are only weakly expressed on the erythrocytes of these patients, they will become quickly sensitized to the Kell antigens that are ubiquitous in the population if they are not transfused with K^x-negative McLeod blood products. Unless the molecular genetic basis of a patient's CGD is known not to involve a deletion of the Xp21.1 region, erythrocyte antigen phenotyping should be done prior to a CGD patient's first blood product transfusion. For patients found to have McLeod syndrome, early consideration should be given to blood storage in case it is needed for surgery or transplantation later on.

Clinically significant or symptomatic complications of granuloma formation are best treated with corticosteroids, as these lesions respond quickly to relatively low doses of these agents (e.g., 0.5–1.0 mg/kg per day of prednisone) (Chin et al., 1987; Danziger et al., 1993). It is usually necessary to treat the granulomas for a few weeks before tapering the steroid dose to prevent a rapid relapse. Corticosteroid therapy (topical or by mouth) may also be required to control the symptoms of CGD inflammatory bowel disease. In general, however, corticosteroid therapy in CGD patients should be avoided whenever possible to minimize the inevitable immunosuppression caused by these agents.

The mechanism of immune pathology in CGD is still opaque. Unrestrained IL-17–producing γδ T-cell activity due to the absence of neutrophil- and macrophage-associated ROS formation and lack of Treg lymphocytes may represent mechanisms in which rhIFN-γ has a role in combination with L-kynurenine derivates. However, the failure to find the human equivalents of the mouse model makes this unlikely. In clinical practice, steroids rescue patients suffering from acute mulch pneumonitis, a syndrome induced by inhalation of large loads of fungal spores, as occurs when gardening or mulching. This syndrome presents with fever, dyspnea, hypoxia, and diffuse infiltrates in the lungs. It is best treated with immediate initiation of corticosteroids (1 mg/kg/d) and antifungals (Siddiqui et al., 2007).

Bone marrow transplantation can cure CGD (see Chapter 60) and has been successfully employed (Fischer et al., 1986; Ho et al., 1996; Kamani et al., 1988). However, the overall results have been mixed, as failure to engraft has been a major problem, at least in the earlier transplants. It appears that newer conditioning regimens employing higher doses of busulfan to achieve adequate myeloid suppression may have overcome this problem (Ho et al., 1996; Seger et al., 2002). A European study reports the outcome of 27 transplanted CGD patients in the period 1985 to 2000. Most of these were children, and most received a myeloablative busulfan-based regimen and had unmodified marrow allografts from HLA-identical sibling donors. After myeloablative conditioning, all patients (23) were fully engrafted with donor cells, whereas after myelosuppressive regimens only 2 of 4 patients were

engrafted (Seger et al., 2002). This latter result recalls a similar observation in the United States (Horwitz et al., 2001). In the European study, severe graft-versus-host disease, exacerbation of infection during aplasia, and an inflammatory flare were seen in the subgroup of nine patients with preexisting infection. Overall survival was 85 percent, with 96 percent cured of CGD. Survival was excellent in patients without infection at the moment of transplantation. Preexisting infections and inflammatory lesions cleared in all engrafted survivors.

Life-threatening bacterial and fungal infections and high level of morbidity with colitis, growth failure, and respiratory disease have led the effort to continue improving transplantation protocols in CGD. A recent UK study (Soncini et al., 2009) contained data from 20 patients; 16 received myeloablative conditioning (busulfan 16 mg/kg, cyclophosphamide 200 mg/kg) with alemtuzumab (CAMPATH-H1; anti-CD52 MoAb, 1 mg/kg) if a non-sibling donor was used. Those receiving unrelated donor transplants did just as well as those receiving matched sibling transplants. Survival was 90 percent, with two deaths due to preexisting disseminated fungal disease. Complications occurred in patients with preexisting infection or inflammation, as would be expected. For the survivors, growth improvement was good and major infections were absent beyond 3 months posttransplantation. Morbidity related to HSCT or graft-versus-host disease in some of the patients was considerable.

The reduced intensity conditioning (RIC) protocol used in Zürich (busulfan 8 mg/kg, fludarabine 180 mg/m^2, alemtuzumab [antiCD52 MoAb] 0.3 mg/kg) has also shown excellent outcome in 10 CGD patients transplanted with HLA-identical siblings, with tolerance of the procedure in all patients. There were a few days of bone marrow aplasia after day +10 but full engraftment in all patients. Again, 90 percent survived. Minor mismatches still matter, but alemtuzumab has been shown to result in very efficient prophylaxis against graft-versus-host diseases. Graft-versus-host disease more than grade 2 was absent even with matched unrelated donors. Secondary tumors are not an issue in primary immunodeficiency in general and have not been seen in the CGD setting, even with myeloablative regimens. Probably, this holds true for the lack of infertility as well (Güngör et al., 2005, 2009).

Knowing that supportive care and the efficacy of newer antifungal drugs will continue to improve outcome, it will remain difficult to advise whether a given CGD patient should undergo bone marrow transplantation in the absence of serious infections or inflammatory disease in the past. The current 10 percent mortality rates will remain an unacceptable obstacle to preemptive transplantation for many parents of young CGD patients. Prognostic indicators such as superoxide production (Kuhns et al., 2010) and disease-modifying genes possibly associated with outcome in CGD (see Phenotype–Genotype Correlation section) may be helpful in making the choice between conservative management and bone marrow transplantation.

FUTURE DIRECTIONS

The improvement in the prognosis for patients with CGD over the past 10 to 15 years has been remarkable. Patients diagnosed in infancy and treated with prophylactic antibiotics, antifungals, and rIFN-γ before a relentless cycle of serious infections begins have a fairly optimistic future. It is clear, however, that the prophylactic and therapeutic approaches currently employed are still imperfect, as even these very young, optimally treated children can develop disastrous infections and die. Moreover, all of the treatments developed to date (with the exception of bone marrow transplantation) are only supportive, not curative, and demand a lifetime of diligence and compliance. While further refinements in the medical and surgical management of CGD are likely in the years to come, the ultimate goal is the development of curative approaches for this immunodeficiency. Attention has appropriately been directed toward gene replacement therapy (see Chapter 61). The study of patients with variant forms of X-CGD has revealed that levels of oxidase activity as low as 3 to 5 percent in neutrophils may be associated with a mild clinical phenotype. In addition, insights derived from X-CGD carriers indicate that as few as 5 percent fully functioning neutrophils can provide substantial protection against infection. It is thus clear that full correction of 100 percent of the circulating neutrophils in CGD patients is not required to ameliorate disease. Even if corrected cells are only transiently generated, there is the potential for substantial benefits for CGD patients, as this therapeutic approach could be used to help treat stubborn, recalcitrant infections.

Partial correction of the oxidase defect has been achieved in vitro by transduction of CD34-positive peripheral blood or bone marrow progenitor cells from patients with any of the four major types of CGD, using retroviral vectors containing the correct cDNA for the missing NADPH oxidase component (Li et al., 1994; Porter et al., 1996; Sekhsaria et al., 1993; Weil et al., 1997). Substantial reconstitution of oxidase activity was also achieved in granulocyte/monocyte bone marrow progenitors from an X-CGD gp91^{phox} knockout mouse by means of a retroviral vector system (Ding et al., 1996). This approach has been extended to the treatment of gene knockout CGD mice lacking either gp91^{phox} or p47^{phox}, in which bone marrow progenitors were treated ex vivo and then transplanted into irradiated syngeneic CGD mice (Björgvinsdóttir et al., 1997; Mardiney et al., 1997). In both cases, the recipient mice showed increased resistance to bacterial and fungal infection. Levels and duration of expression of the transduced oxidase subunits have been sufficiently large to justify the initiation of early clinical trials for these two forms of CGD (Malech et al., 1997).

Unfortunately, to date the results have been disappointing. In five p47^{phox}-deficient CGD patients, the fraction of gene-corrected neutrophils in the circulation was never more than 0.05 percent, and corrected cells were no longer detectable after 6 months (Malech et al., 1997). Similar results were obtained in two X-CGD patients (Malech et al., 1999). Several problems became evident: the corrected bone marrow cells had no growth advantage over the uncorrected cells (so bone marrow conditioning was likely necessary), the expression of the transgene was very weak (so the transgene cDNA needed to be optimized), and the transgene could be silenced because of its unnatural

promoter (so vectors were needed with the original myeloid promoter sequence).

A more recent clinical trial was performed with a spleen focus-forming γ-retroviral vector (Ott et al., 2006). Two adult X-CGD patients were conditioned with a submyeloablative dose of liposomal busulfan before reinfusion of the transduced (40–45 percent transduction efficiency) granulocyte colony-stimulating factor (G-CSF)-mobilized $CD34^+$ cells. The patients clinically improved within 2 months: in one patient, a chronic liver abscess resolved, and the other achieved resolution of a fungal lung infection. In both patients a limited expansion in the number of gene-corrected cells from initially 10 to 30 percent to 40 to 60 percent was observed starting 5 months after transplantation. The expansion was the result of activating retroviral insertions into three proto-oncogenes, *MDS1/EVI1*, *PRDM16*, and *SETBP1*. Thereafter, silencing of the $gp91^{phox}$ transgene expression took place, leading to barely detectable O_2^- generation by the gene-corrected cells, and death of one patient from severe sepsis with multiple organ failure (Seger, 2008). Therefore, gene therapy for CGD patients is at present only an option to overcome very recalcitrant infections, before allogeneic bone marrow transplantation can be performed.

The risk of insertional mutagenesis and transactivation of proto-oncogenes from retrovirus-mediated gene therapy necessitates the development of vectors with improved safety. Self-inactivating (SIN) vectors lacking the potent retroviral enhancer elements within the long terminal repeats (LTR) show much less transactivation potential than conventional LTR-driven vectors (Modlich et al., 2006). Transgene ($gp91^{phox}$) expression in SIN vectors is driven by an internal, tissue (myelocyte)-specific, cellular promoter, further reducing the probability of oncogene activation at the stem-cell level. Optimization of the transgene cDNA has led to increased vector titers and transgene expression (Moreno-Carranza et al., 2009). Such vectors will soon be used in clinical trials with CGD patients. Lentiviral vectors could offer additional safety by integrating into transcriptional units, as opposed to γ-retroviral vectors integrating in proximity to promoter regions. Moreover, lentiviral vectors allow gene transfer into quiescent cells and do not require extensive preculture of CD34-positive cells with cytokines. Preclinical testing for lentiviral gene therapy trials in CGD is ongoing (Roesler et al., 2002; Santilli et al., 2011).

Two recent developments indicate important progress in this area. One is the epigenetic reprogramming of somatic cells from mammals into induced pluripotent stem cells (iPS cells) (Takahashi & Yamanaka, 2006). Most often, the starting material is skin fibroblasts, but blood cells or bone marrow cells can also be used (Ye et al., 2009; Zou et al., 2011). In the original publication, these cells were then transfected with four vectors, containing cDNA for the transcription factors Oct3/4, Sox2, c-Myc, and Klf4, respectively, and cultured under embryonic stem-cell conditions. In later studies, these four vectors have been combined into one polycistronic lentiviral cassette, which can be excised after a few days so as to leave no permanent changes in the genome (Somers et al., 2009, 2010). The iPS cells possess a high reproduction capacity and can be induced to differentiate into all different somatic cells, including myeloid cells (Morishima et al., 2011; Mukherjee et al., 2011; Zou et al., 2011). This system works with human cells as well as with other mammals, thus creating the possibility to create a repository of patient-derived iPS cells. Indeed, iPS cells and somatic cells derived from iPS cells contain the same DNA mutations as do the original somatic cells (Jiang et al., 2012; Mukherjee et al., 2011; Pessach et al., 2011; Zou et al., 2011). For research purposes one can think of limitless numbers of patient neutrophils, patient myeloid precursor cells that can be transfected before differentiation into neutrophils, or generation of other somatic cells that may express the mutated protein. For treatment purposes one can think of restoring NADPH oxidase capacity in the patient's own derived hematopoietic precursor cells and transplantation of these autologous cells into the patients, or production of large numbers of autologous functional neutrophils for transfusion purposes. One unsolved question at this moment is whether the specificity and extent of all epigenetic control mechanisms in the original blood neutrophils are identical to neutrophils derived in vitro from iPS cells.

A second important new development is the progress made in gene correction—that is, replacement of that region of a gene that carries a mutation by its wild-type counterpart while leaving all neighboring sequences intact. This strategy has the inherent advantage of leading to correct protein expression under the control of the endogenous promoter and enhancer regulatory elements. This strategy was first attempted by Cole-Strauss et al. (1996) and Kren et al. (1997) by chimeric RNA/DNA oligonucleotide transfection of cultured lymphoblasts and human hepatoma cells, and later in vivo in rats (Kren et al., 1998). The oligonucleotide chimera anneals efficiently and specifically to the target sequence in the gene, causing mutagenesis (or mutation correction) by DNA repair. The chimera is delivered as a complex with a protecting polycation that in turn is modified with a ligand to promote targeting to a specific receptor (e.g., the asialoglycoprotein receptor of hepatocytes). Despite efficient mutagenesis in the rat liver, this system has not gained wide application in human gene correction. Instead, gene correction through introducing site-specific double-strand DNA breaks by engineered homing endonucleases (also called meganucleases) (Munoz et al., 2011) or zinc-finger nucleases (Nakayama, 2010; Urnov et al., 2005; Zou et al., 2011), followed by homologous recombination of the mutated gene region with a similar exogenous wild-type cDNA source through endogenous DNA repair mechanisms, has been developed. The introduction of site-specific double-strand DNA breaks greatly enhances correction efficiency and has also been shown in vivo in a hemophilia B mouse model to be sufficiently effective to restore hemostasis through 2 to 3 percent correction of circulating factor IX expression (Li et al., 2011). For gene correction of CGD this may not be the method of choice, given that many CGD patients suffer from disease caused by unique mutations. This would imply engineering homing endonucleases or zinc-finger nucleases for each patient separately. Instead, targeting specific so-called safe harbors in the genome whose disruption does not lead to deleterious consequences such as insertional mutagenesis may be an attractive alternative. One such locus, the AAVS1 locus (the common

integration site of the adeno-associated virus 2), has been used for lentiviral transduction of CGD iPS cells (Zou et al., 2011). This last approach of course lacks the attractive prospect of gene correction in situ. Although this all sounds promising, a number of problems remain to be solved. One is the risk of undesired toxicity as a result of off-target cleavage by the nucleases, which may lead to break-induced DNA sequence alterations caused by nonhomologous end-joining as well as chromosomal translocations possibly leading to oncogenic transformation. Cellular delivery of the nucleases must also be considered. Use of retrovirus or integrating lentivirus may reintroduce the risk of insertional mutagenesis, and prolonged expression of the cDNA for the nucleases may cause deleterious effects as mentioned. Therefore, nonintegrating lentivirus (NILV) vectors that allow expression only for a limited time appear attractive, although such vectors allow less efficient expression than the integrating vectors (Pessach & Notarangelo, 2011).

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53.

CELL ADHESION AND LEUKOCYTE ADHESION DEFECTS

Amos Etzioni and Ronen Alon

Leukocyte trafficking from bloodstream to tissue is important for the continuous surveillance for foreign antigens and for rapid leukocyte accumulation at sites of inflammatory response or tissue injury. Leukocyte emigration to sites of inflammation is a dynamic process, involving multiple steps in an adhesion cascade. These steps must be precisely orchestrated to ensure a rapid response with only minimal damage to healthy tissue (Carlos & Harlan, 1994; Ley et al., 2007; Springer, 1994; von Andrian & Mackay, 2000). Leukocyte interaction with vascular endothelial cells is mediated by several families of leukocyte and endothelial adhesion molecules, including the integrins, the selectins, and members of the immunoglobulin gene superfamily (IgSF). Each is involved in a different phase of leukocyte adherence to and migration across the endothelium, and the synchronization of their surface expression and activation is crucial for leukocyte trafficking.

While the importance of leukocyte movement toward sites of inflammation was well recognized more than a century ago, the molecular basis of leukocyte emigration from the bloodstream through the endothelium was only recently elucidated. The integrin family and several integrin ligands of the IgSF were described in the 1980s, whereas the selectins and their carbohydrate counter structures were discovered in the 1990. The critical role of various cytoplasmatic adaptors for integrin activation was realized only recently (Legate & Fassler, 2009). Several years after reporting the structure of the leukocyte integrin molecule, a genetic defect in the subunit of the molecule (ITGB2) was discovered (Springer et al., 1984). The resultant syndrome, now called leukocyte adhesion defect type I (LAD I; OMIM 116920), has been described in more than 250 children and is characterized by delayed separation of the umbilical cord, recurrent soft tissue infections, chronic periodontitis, marked leukocytosis, and a high mortality rate at an early age. Currently, the only definite therapy is bone marrow transplantation. In vivo and in vitro studies have shown a marked defect in neutrophil motility.

In 1992 a second defect, LAD II (OMIM 266265), was discovered in two children and was found to be due to a defect in the synthesis of selectin ligands (Etzioni et al., 1992). Although several adhesive functions of LAD II leukocytes are markedly impaired in vitro, the clinical course with respect to infectious complications is milder than that in LAD I. However, patients with LAD II present with other abnormal features, such as growth and mental retardation, which are related to the primary defect in fucose metabolism and are not observed in LAD I.

Recently, a third rare LAD syndrome has been described (Alon et al., 2003; Alon & Etzioni, 2003; Kuijpers et al., 1997a, 2009; Malinin et al., 2009; Svensson et al., 2009). Patients with LAD III (OMIM 612840) suffer from severe recurrent infections, similar to LAD I, but also have a severe bleeding tendency, resembling Glanzmann's thrombasthenia. Although integrin expression is normal and its structure is intact, a defect in inside-out integrin activation on all hematopoietic cells is the primary abnormality in LAD III. Finally, several homozygous stop codon mutations in Kindlin-3 were found in patients with the LAD III phenotype (Kuijpers et al., 2009; Malinin et al., 2009; Mory et al., 2008; Svensson et al., 2009).

In this chapter we will briefly review the various adhesion molecules involved in leukocyte–endothelial interactions and then focus on the clinical consequences of defects in these molecules.

ADHESION MOLECULES

LEUKOCYTE INTEGRINS

Integrins are transmembrane cell-surface proteins that bind externally to matrix and membrane proteins and internally to

cytoskeleton proteins and thereby communicate between the extracellular and the intracellular environments of the cell (Luo et al., 2007). Each integrin consists of noncovalently linked α and β subunits. Each subunit is composed of a large extracellular domain (1,000 to 1,150 amino acids for the α subunit and 700 to 740 for the β subunit), a single transmembrane domain of about 20 amino acids, and a short cytoplasmic domain of 40 to 50 amino acids (Askari et al., 2009; Humphries, 2000). Specificity of receptor binding to various ligands appears to depend primarily on the extracellular portion of the α subunit, although both subunits are required for functional activity of the receptor.

Integrins have been arranged in subfamilies according to the 3 main β subunits they consist of, and each of these β subunits can associate with 1 to 10 different α subunits. To date, there are 19 α- and 8 β-subunit genes identified in mammals, encoding polypeptides that combine to form up to 25 integrin heterodimers. Within this large family of integrin receptors only four members have been shown to be significantly involved in leukocyte adhesion to endothelium.

Two, LFA-1 and Mac-1, belong to the β_2 subfamily, and the other two, VLA-4 and $\alpha_4\beta_7$, belong to the β_1 and the β_7 subfamilies, respectively.

β2 Integrins

This subfamily comprises four known heterodimeric glycoproteins with a common β subunit, designated CD18 (Table 53.1). The first three heterodimers identified were lymphocyte function-associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1), and p150, 95; their α subunits are designated α_L (CD11a), α_M (CD11b), and α_X (CD11c), respectively. A fourth heterodimer, $\alpha_D\beta_2$, was subsequently identified. The α_L, α_M, α_X, α_D, and β_2 polypeptides are initially assembled in the cytoplasm, and the mature integrin heterodimer is then transported to the plasma membrane. Both subunits are therefore required for integrin expression on the plasma membrane.

β_2 integrin expression is restricted to leukocytes and to subsets of mesenchymal stem cells (Ley, 2002), but different heterodimers are differentially expressed on different subsets of leukocytes. Whereas LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) is expressed by all leukocytes, Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) and p150, 95 ($\alpha_X\beta_2$, CD11c/CD18) are expressed primarily by myeloid cells, subsets of lymphocytes, and natural killer (NK) cells. $\alpha_D\beta_2$ (CD11d/CD18) is expressed at moderate levels on myelomonocytic cell lines and subsets of peripheral blood leukocytes. Importantly, the β_2 integrins participate in many cellular contacts essential to various immune effector functions in addition to mediating leukocyte adherence to endothelium. These include phagocytosis, killing of bacteria, antibody-dependent cell-mediated cytotoxicity, antigen stimulation, and both homotypic and heterotypic aggregation.

An important characteristic of the leukocyte β_2 integrins is that under baseline conditions they exist in a relatively inactive nonadhesive conformation. One of the key events in leukocyte adhesion to endothelium is the activation and deactivation of these integrins at the appropriate time and place. Activation of leukocytes by a variety of mediators (e.g., for phagocytes, C5a, platelet-activating factor, leukotriene B4, or interleukin (IL)-8; for lymphocytes, chemokines and antigens) results in a transient increase in adhesion by CD11/CD18-dependent mechanisms. This increased adhesiveness occurs through qualitative changes, involving transformation of LFA-1 and Mac-1 from a low- to high-affinity state and a subsequent clustering of receptors, which increases the avidity of the cell contact (Ley, 2002). The main β_2 integrin ligands on vascular endothelium are members of the immunoglobulin superfamily (IgSF). Aside from its role in leukocyte adhesion, occupancy of CD11/CD18 by various ligands also induces intracellular signals. Thus, ligand binding could affect cellular functions such as survival, differentiation, apoptosis, cytotoxicity, proliferation, and cytokine production (Luster et al., 2005).

α_4 Integrins

β_1 or very late antigen (VLA) integrins represent the largest integrin subfamily (Table 53.1). Most of these integrins are receptors for extracellular matrix proteins, including fibronectin, collagen, and laminin. One member of this subfamily, $\alpha_4\beta_1$ (VLA-4; CD49d/CD29), is involved in lymphocyte, eosinophil, basophil, NK cell, hematopoietic stem cell, and monocyte adhesion to the CS-1 fragment of fibronectin and to the cytokine-induced endothelial cell-surface protein, vascular cell adhesion molecule-1 (VCAM-1). Circulating human neutrophils do not normally express significant levels of VLA-4 and do not use this pathway to adhere to activated endothelium; they rely almost exclusively on their β_2 integrins for stable interactions with vascular endothelia (Carman & Springer, 2003).

The $\alpha_4\beta_7$ integrin is expressed on lymphocytes and eosinophils and functions as the homing receptor to vascular beds enriched with the mucosal vascular addressin cell adhesion molecule (MAdCAM-1) (Table 53.1). A subset of activated $\alpha_4\beta_7$ can also bind, although weakly, to the VLA-4 ligands, VCAM-1 and fibronectin.

IMMUNOGLOBULIN SUPERFAMILY MOLECULES

Intercellular Adhesion Molecules

The intercellular adhesion molecules (ICAMs), of which five have been identified, were originally defined as LFA-1 ligands (Springer, 1994) (Table 53.1). Only ICAM-1 and ICAM-2 are expressed on endothelium and participate in leukocyte adhesion to endothelium. The gene for ICAM-1 is located on chromosome 19. The molecule has five Ig-like domains, with a short hinge region separating the third and fourth Ig-like domains (Luster et al., 2005). ICAM-1 is a ligand for both LFA-1 and Mac-1. The binding sites in ICAM-1 for the two integrins are, however, distinct; LFA-1 binds to domains 1 and 2 and Mac-1 binds to domain 3. Human ICAM-2 is a single-copy gene located on chromosome 17. It has only two extracellular Ig-like domains, and the binding site for LFA-1 is located in these domains (Ley et al., 2007). ICAM-2 binds LFA-1 with a lower affinity than ICAM-1. The other β_2 integrins do not bind ICAM-2.

Table 53.1 ADHESION MOLECULES INVOLVED IN LEUKOCYTE–ENDOTHELIAL INTERACTIONS

NAME	DISTRIBUTION	MEDIATORS (INDUCED BY; ACTIVATED BY)	COUNTERSTRUCTURE(S)	FUNCTION(S)
Integrins				
LFA-1 ($\alpha_L\beta_2$, CD11a/CD18)	All leukocytes	Constitutively expressed; activated by cross-linking of other surface receptors, chemokines	ICAM-1, 2	Leukocyte firm adhesion and transmigration
Mac-1 ($\alpha_M\beta_2$, CD11b/CD18)	Neutrophils, monocytes, natural killer cells, eosinophils	Constitutively expressed; activated by cytokines, chemokines, chemoattractants	ICAM-1	Phagocyte firm adhesion and transmigration
$\alpha_4\beta_7$	Lymphocytes, eosinophils, natural killer cells	Constitutively expressed; activated by chemokines	MAdCAM-1, VCAM-1, fibronectin	Leukocyte rolling and firm adhesion
VLA-4 ($\alpha_4\beta_1$, CD49d/CD29)	All leukocytes except neutrophils	Constitutively expressed; activated by cross-linking of other surface receptors, chemokines, chemoattractants	VCAM-1, fibronectin	Leukocyte rolling, firm adhesion, and migration
Ig Superfamily ICAM-1 (CD54)	Endothelial cells, lymphocytes, other cell types	Constitutively expressed; upregulated by IL-1, LPS, TNF, IFN-γ	LFA-1, Mac-1	Leukocyte firm adhesion and transmigration
ICAM-2 (CD102)	Endothelial cells,	Constitutively expressed	LFA-1	Leukocyte firm adhesion
VCAM-1 (CD106)	leukocytes Endothelial cells, other cell types	Induced by TNF IL-1, LPS, etc.	VLA-4, $\alpha_4\beta_7$	Leukocyte rolling and firm adhesion
PEC AM-1 (CD31)	Leukocytes, endothelial cells, platelets	Constitutively expressed	PECAM-1(CD31)	Transmigration
MAdCAM-1	Mucosal HEV	Constitutively expressed	$\alpha_4\beta_7$, L-selectin	Leukocyte rolling and firm adhesion
Selectins				
E-selectin (CD62E)	Endothelial cells	Induced by TNF, IL-1, LPS, etc.	Sialylated, fucosylated structures (e.g., SLeX) expressed on PSGL-1, and a variety of other glycoproteins	Leukocyte rolling
P-selectin (CD62P)	Endothelial cells, platelets	Induced by histamine, thrombin, etc.	Sialylated, fucosylated, sulfated structures on leukocyte surfaces	Leukocyte rolling
L-selectin (CD62L)	Most leukocytes	Constitutively expressed; shed by diverse activating agents	CD34, PSGL-1 (sialyated, fucosylated and sulfated) structures expressed on CD34, MAdCAM-1, GlyCAM-1, PSGL-1, and other glycoproteins	Leukocyte rolling

ICAM-1 is expressed usually at low levels on most endothelial cells but can be dramatically upregulated upon endothelial activation by lipopolysaccharides (LPS), IL-1, and tumor necrosis factor (TNF). This increased expression of ICAM-1 peaks at 4 to 6 hours and persists for days with continued exposure to cytokines. ICAM-2 expression, in contrast, is expressed constitutively and is not regulated by cytokines.

VCAM-1

VCAM-1 also belongs to the IgSF. A single VCAM-1 gene gives rise through alternative splicing to distinct isoforms that differ in the number of integrin binding sites (Wittchen, 2009). In humans the predominant isoform contains seven Ig-like domains with binding sites for the integrin VLA-4 located within the first and fourth domains (Table 53.1). VCAM-1 is induced by cytokines on endothelial cells with a time course similar to that of ICAM-1.

MAdCAM-1

MAdCAM-1 is a member of the IgSF that has a mucin-like domain and is a high-affinity ligand for the $\alpha_4\beta_7$ integrin (Briskin et al., 1993) (Table 53.1). A subset of MAdCAM-1 expressed on high endothelial venules (HEVs) is decorated with carbohydrate ligands for L-selectin, MAdCAM-1 may serve as a bridge between selectin- and β_2 integrin–dependent events, especially in mucosal tissues and gut-associated lymphoid organs (Bargatze et al., 1995).

Platelet–Endothelial Cell Adhesion Molecule-1

Platelet–endothelial cell adhesion molecule-1 (PECAM-1; CD31) is a member of the IgSF that is expressed at low levels on most leukocytes and platelets but at high levels on endothelial cells, where it is localized to intercellular junctions (Table 53.1). PECAM-1 is involved in paracellular transendothelial migration and can also regulate leukocyte migration through the subendothelial basement membrane (Sperandio et al., 2003).

SELECTINS

This family of adhesion molecules was discovered in 1989, when the cDNA sequences for three distinct cell-surface glycoproteins found on endothelium (E-selectin, CD62E), platelets (P-selectin, CD62P), and leukocytes (L-selectin, CD62L) were reported (McEver, 2002). These molecules were initially designated ELAM-1; PADGEM or GMP-140; and MEL-14 or LECAM-1, respectively. The genes for the selectins are closely linked on chromosome 1, reflecting their common evolutionary origin. All three members have common structural features, most prominently an N-terminal lectin-like domain that is central to the carbohydrate-binding properties of all three selectins (Kansas, 1996). The lectin domain is followed by a regulatory domain homologous to the epidermal growth factor (EGF), which does not interact with ligand but can modulate the ligand-binding properties of the lectin domain, and by a discrete number of domains similar to those found in certain complement binding-proteins. The term *selectin* was proposed to highlight the lectin domain that is critical for ligand binding by all selectins and to indicate the selective functions and expression patterns of these cell adhesion lectins.

Although three selectins are involved in the recruitment of leukocytes to sites of tissue injury, there are fundamental differences in their distribution, activation, and mode of expression (McEver, 2002). For example, while all three selectins take part in the first step of the adhesion cascade, the capture and rolling phase (vide infra), E-selectin participates also in slowing down rapidly rolling leukocytes.

E-selectin

E-selectin expression is restricted to endothelial cells and its transcription is induced when endothelial cells are activated by LPS, IL-1, TNF, and other stimuli (Table 53.1). These substances elevate the translation of E-selectin, resulting in peak cell-surface expression at 4 to 6 hours, decreasing to near basal levels by 24 hours (Cotran et al., 1986). Although E-selectin expression in vitro is typically transient, it is expressed chronically in certain inflammatory conditions.

P-selectin

P-selectin, expressed on platelets as well as on endothelial cells (Table 53.1), does not necessarily require de novo synthesis because it is stored in secretory granules (Weibel-Palade bodies). Thus, within seconds of activation by thrombin or histamine, P-selectin is rapidly redistributed to the cell surface. Its expression in vitro is typically very short-lived—up to 15 minutes. However, studies in vivo suggest that endothelial P-selectin is also upregulated by inflammatory stimuli at the level of de novo transcription and protein synthesis, providing a mechanism for more prolonged expression (Siegel-Axel & Gawaz, 2007).

L-selectin

In contrast to the E- and P-selectins, L-selectin is not expressed on endothelial cells or platelets but rather is constitutively expressed on leukocytes (Table 53.1). Although originally described as a lymphocyte homing receptor, it was subsequently shown to be expressed on most other leukocytes (Rosen, 2004). L-selectin can be shed rapidly, mainly in neutrophils via proteolytic cleavage of its ectodomain near the membrane insertion. Cleavage of L-selectin may serve to limit further leukocyte recruitment at inflammatory sites but may also facilitate integrin turnover during transendothelial lymphocyte migration.

SELECTIN LIGANDS

Studies of the molecular basis of selectin adhesion have focused mainly on carbohydrate recognition by the lectin domains. Identification of the physiological ligands for the selectins has been challenging because, like many lectins, the

selectins can bind a variety of carbohydrate structures in vitro (Rosen 2004). These ligands constitute the termini of either N-linked or O-linked glycans, which can be part of multiple glycoprotein scaffolds.

All selectin carbohydrate ligands share a core of sialylated and fucosylated lactosamine tetrasaccharides related to the sialylated Lewis X blood group (SLeX, CD15). SLeX is expressed at high levels on neutrophils and monocytes, whereas most peripheral lymphocytes express SLeX only after activation and exposure to specific cytokines. Both the sialic acid and the fucose moieties are critical for efficient binding to E-selectin, while additional sulfate groups on the glycan or the glycoprotein confer binding to L-selectin and P-selectin. Monoclonal antibodies against multiple SLeX-containing glycan derivatives and infusion of soluble SLeX can both attenuate the binding of neutrophils to activated endothelial cells, as well as leukocyte rolling along inflamed vessels (Asako et al., 1994). SLeX and selectin binding can be introduced to many cell types via transfection of appropriate fucosyltransferases. While the selectins bind weakly to small sialylated, fucosylated oligosaccharides, they appear to bind with much higher avidity to carbohydrate determinants presented by a limited number of glycoproteins. L-selectin binds at least five different heavily glycosylated mucin-like proteins: GlyCAM-1, CD34, and related glycoproteins like podocalyxin and endoglycan, and the integrin ligand MAdCAM-1 (Rosen, 2004). P-selectin and, to a lesser extent, E-selectin have been reported to selectively bind P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed on most leukocytes and can also serve to capture leukocytes onto endothelial adherent leukocytes via L-selectin–dependent interactions. A monoclonal antibody specific for PSGL-1 completely inhibited P-selectin–mediated rolling of leukocytes under a range of physiological shear stresses (Moore et al., 1995).

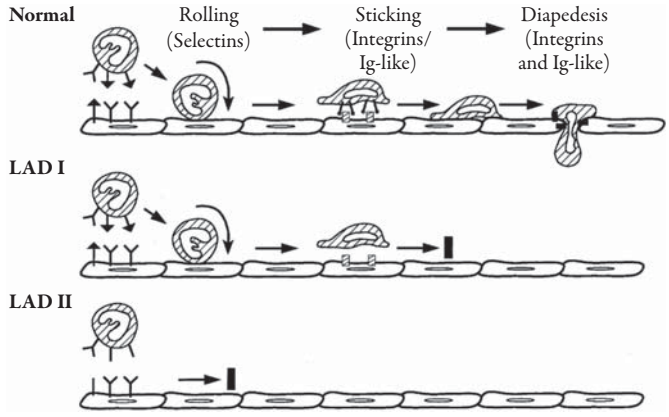

Figure 53.1 The adhesion cascade and LAD syndromes. Under conditions of flow at sites of inflammation, normal leukocytes first roll along the venular wall via selectin–carbohydrate interactions. Following integrin activation by local chemoattractants or chemokines, the rolling cells then adhere firmly via binding of leukocyte integrins to endothelial IgSF ligands. Subsequently, the adherent leukocytes diapedese between endothelial cells utilizing PECAM-1 and integrin–IgSF ligand interactions. Defects in selectin-mediated rolling or integrin-mediated sticking impair emigration. In LAD I, neutrophils deficient in β_2 integrin can roll along the endothelium but cannot adhere firmly to transmigrate. In LAD II, neutrophils deficient in fucosylated carbohydrate ligands for selectins are unable to roll and thus cannot adhere and transmigrate.

THE ADHESION CASCADE

The migration of leukocytes from the bloodstream to the tissue occurs in several distinct steps (Ley et al., 2007) (Fig. 53.1). First, under conditions of flow, loose adhesions generated between the leukocyte and the vessel wall, primarily in postcapillary venules, reversibly capture flowing leukocytes and mediate their rolling on the endothelium. This transient and reversible step is a prerequisite for activation of leukocytes by specific endothelial displayed chemotactic cytokines (Laudanna et al., 2002). This is followed by firm adhesion (i.e., sticking), after which migration occurs. Each of these steps involves different adhesion molecules and can be differentially regulated.

SELECTIN-MEDIATED CAPTURE AND ROLLING

Leukocytes that adhere to blood vessels must resist shear forces exerted on them as they interact with the vascular endothelium. Under normal conditions, leukocytes move rapidly in the laminar flow stream of blood and do not adhere to the endothelium, except for subsets of venules within lymphoid organs, where constitutive extravasation of lymphocytes must take place (Wagner & Frenette, 2008). However, with activation of essentially all peripheral postcapillary venules due to local trauma or inflammation, leukocytes immediately begin to roll along the venular wall. This phenomenon of leukocyte rolling was observed for more than a century, but its molecular basis was delineated only two decades ago. Several studies showed that although CD18-blocking antibodies interfere with leukocyte sticking, they do not prevent rolling. Monoclonal antibodies to selectins, however, markedly reduced the rolling process both in vivo and in vitro. Moreover, leukocyte rolling is diminished in P-, E-, and L-selectin–deficient mice (Mayadas et al., 1993). Patients who lack SLeX molecules on their leukocytes and mice deficient in the enzymes essential for generating this motif also show defective in vivo rolling (Homeister et al., 2001; von Andrian et al., 1993). L-selectin itself recognizes various sulfated derivatives of the SLeX moiety, which are associated with multiple glycoproteins on HEVs (Baumheter et al. 1993), as well as with as yet poorly characterized glycoproteins expressed on cytokine-stimulated systemic vasculature.

ACTIVATION OF LEUKOCYTE INTEGRINS

The initial rolling interaction of leukocytes with endothelium is reversible unless the leukocytes are activated and become firmly adherent via integrins. Rolling mediated by selectins brings the leukocytes in close proximity to endothelial displayed chemoattractants (Alon & Rosen, 2007). These chemotactic cytokines made by the endothelium or by cells in the surrounding tissue can rapidly switch the otherwise

nonadhesive leukocyte integrins into highly adhesive molecules that mediate leukocyte sticking and firm arrest. The leukocyte chemoattractants include classical chemotactic reagents such as C5a and leukotriene B4, and chemokines, which are stably tethered on glycosaminoglycans (GAGs) expressed on the apical surface of the endothelial cells (Mackay, 2001). The importance of these chemotactic agents in mediating firm adhesion was illustrated in experiments using the flow chamber model. In this model rolling leukocytes stop immediately on the β_2 integrin ligand ICAM-1 only upon exposure to a chemotactic agent (Lawrence & Springer, 1991). Rolling is not a prerequisite for integrin activation since lymphocytes encountering α_4 ligands such as VCAM-1 and a co-immobilized chemokine can immediately arrest on this ligand without prior rolling (Grabovsky et al., 2000). Increased adhesive capacity of leukocyte integrins is mediated primarily through local changes in integrin receptor affinity as well as in avidity. In phagocytes, a rapid increase in surface expression of the integrin Mac-1 also occurs through translocation of $\alpha_M\beta_2$ heterodimers from cytoplasmic granules to the plasma membrane.

ADHESION AND TRANSENDOTHELIAL MIGRATION VIA INTEGRINS AND IGSF LIGANDS

Activation of integrins results in increased binding to their Ig-like ligands on the endothelial cells, which allows leukocytes to withstand the continuous shear forces in the blood vessels. Once arrested, the leukocyte must crawl variable distances on the apical and basolateral surfaces of the endothelial lining of blood vessels. This crawling is also mediated by integrins, which form and break serial adhesive contacts (Shulman et al., 2009). Consequently, transendothelial migration of arrested leukocytes to various sites of inflammation depends on multiple integrin–IgSF interactions. Other endothelial expressed members of the endothelial IgSF, such as JAMs, PECAM-1, VE-Cadherin, and CD99, contribute to various steps of migration and also stimulate signals necessary for leukocyte crossing through subendothelial basement membrane (Carman et al., 2007). Nevertheless, whereas diapedesis of myeloid cells depends on these multiple adhesive cues, the transendothelial migration of lymphoid cells does not depend on these signals.

ADHESION MOLECULE DEFICIENCY SYNDROMES

Perhaps the best way to appreciate the in vivo importance of leukocyte-endothelial adhesion molecules and their cytoplasmic regulators in humans is to examine those rare "experiments of nature" in which a genetically determined defect in a particular adhesion pathway is associated with a defined immunodeficiency. Three such rare syndromes have been so far recognized (Hanna & Etzioni, 2012) (Table 53.2). In the first LAD syndrome (LAD I), the β_2 integrin is deficient, whereas in the second, LAD II, the fucosylated carbohydrate ligands for selectins are absent (Etzioni, 1996). Most recently, a third leukocyte adhesion deficiency, LAD III, has been identified in which the key integrin coactivator Kindlin-3 is diminished, resulting in a general defect in both leukocyte and platelet integrin activation and firm adhesiveness (Alon & Etzioni, 2003; Malinin et al., 2009; Svensson et al., 2009).

LAD I

Several reports published before 1980 documented a group of patients with recurrent bacterial and fungal infections, defective leukocyte motility and phagocytosis, impaired wound healing, and delayed separation of the umbilical cord (Boxer et al., 1974; Hayward et al., 1979). In 1980, a seminal study provided a possible molecular basis for this emerging syndrome (Crowley et al., 1980), suggesting that the absence of a high-molecular-weight neutrophil membrane glycoprotein in a young boy was directly responsible for an inherited defect in neutrophil adhesion. This abnormality was manifested in vitro by the failure of leukocytes to spread on surfaces and by impaired chemotaxis. In 1982 the association of this syndrome with a missing membrane glycoprotein was confirmed in two other patients with similar symptoms (Bowen et al., 1982).

Table 53.2 **LAD SYNDROMES**

	LAD I	LAD II	LAD III
Clinical Manifestation			
Recurrent severe infections	+++	+	+++
Neutrophilia			
Basal	+	+++	++
With infection	+++	+++	+++
Periodontitis	++	++	?
Skin infection	++	+	++
Delayed separation of the umbilical cord	+++	-	++
Developmental abnormalities	-	+++	
Bleeding tendency	-	-	+++
Laboratory Findings			
CD18 expression	Low or absent	NL	NL
SLeX expression	NL	Absent	NL
Neutrophil motility	↓↓↓	↓↓	↓↓
Neutrophil rolling	NL	↓↓↓	NL
Neutrophil adherence	↓↓↓	↓	↓↓↓
Opsonophagocytic activity	↓	NL	NL
T and B cell function	↓	NL	Impaired memory?

NL, normal.

At the same time, in separate studies, other investigators identified a new leukocyte membrane glycoprotein heterodimeric complex involved in multiple adhesion-related functions. Mac-1 and LFA-1 were identified in 1979 and 1981, respectively (Anderson & Smith, 2001). A third complex belonging to the same integrin family, p150, 95, was described several years later. A relationship between the inherited defect of neutrophil adhesion and these glycoproteins was suggested by the fact that monoclonal antibodies to the α or β chains of this complex in normal leukocytes induced in vitro defects in chemotaxis and phagocytosis similar to those observed in affected patients. Indeed, in 1984, using specific monoclonal antibodies, multiple groups reported that leukocytes of patients with this syndrome were deficient in expression of both the common β subunit as well as in the various α subunits. These findings led to the proposal that the primary defect in this syndrome is related to mutations in the β_2 subunit and that biosynthesis of the β_2 subunit is necessary for surface expression of the α subunits (Springer et al., 1984).

While many names had previously been given to this syndrome, in the interest of brevity and comprehensiveness, the term *leukocyte adhesion deficiency* (LAD) was proposed in 1987 (Anderson & Springer, 1987). LAD I (OMIMIL6920) is inherited in an autosomal recessive manner since heterozygote carriers of the defective β_2 gene exhibit no significant clinical manifestations.

Clinical and Pathological Manifestations

The prominent clinical feature of these patients is recurrent bacterial infections, primarily localized to skin and mucosal surfaces. These infections are indolent and necrotic and tend to recur. Sites of infection often progressively enlarge, and they may lead to systemic spread of the bacteria. Infections are usually apparent from birth onward, and a common presenting infection is omphalitis with delayed separation of the umbilical cord (Fig. 53.2, Color Plate 53.I). However, not all patients with LAD I have delayed separation of the cord. The most frequently encountered bacteria are *Staphylococcus aureus* and gram-negative enteric organisms, but fungal infections are also common. The absence of pus formation at the site of infection is one of the hallmarks of LAD I. Severe gingivitis and periodontitis are major features among all patients who survive infancy. Impaired healing of traumatic or surgical wounds is also highly characteristic of this syndrome.

The recurrent infections observed in affected patients result from a profound impairment of leukocyte mobilization into extravascular sites of inflammation. Skin windows yield few, if any, leukocytes, and biopsies of infected tissues demonstrate inflammation totally devoid of neutrophils. These findings are particularly striking considering that marked peripheral blood leukocytosis (5 to 20 times normal values) is consistently observed during infections. In contrast to the difficulties experienced in defense against bacterial and fungal microorganisms, LAD I patients do not exhibit increased susceptibility to viral infections, suggesting that lymphocyte extravasation, as opposed to myeloid leukocyte trafficking to sites of infection, is not impaired. Lymphoid tissue is, however, severely depleted of lymphocytes, indicating a role for LFA-1 in lymphocyte homing with possible compensation from α_4 integrins, which are not affected in this disease.

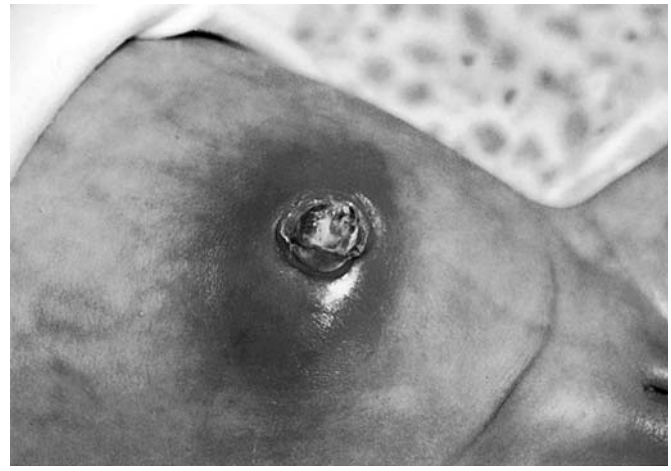

Figure 53.2 Omphalitis in a child with the severe phenotype of LAD I. (See Color Plate.)

Notably, the severity of clinical infectious complications among patients with LAD I appears to be directly related to the degree of CD18 deficiency. Two phenotypes, designated *severe deficiency* and *moderate deficiency*, have been defined (Anderson & Smith, 2001). Patients with less than 1 percent of the normal surface expression exhibit a severe form of disease with earlier, more frequent, and more serious episodes of infection, often leading to death in infancy, whereas patients with some surface expression of CD18 (2.5 to 10 percent) manifested a moderate to mild phenotype with fewer serious infectious episodes and survival into adulthood.

The hallmark pathological finding in severe LAD I is the complete absence of neutrophils in sites of infection. The necrotic areas are devoid of pus. While this finding is typical for many tissues, neutrophil migration to infected lungs may be normal, as a robust inflammatory response has been observed in lung tissue.

Laboratory Findings

The defective migration of neutrophils from patients with LAD I was observed in studies in vivo as well as in vitro (Bowen et al., 1982; Harlan, 1993). Neutrophils fail to mobilize to skin sites in the in vivo Rebuck skin-window test. In vitro studies demonstrate a marked defect in random migration as well as chemotaxis to various chemoattractants. Adhesion and transmigration through endothelial cells are severely impaired (Harlan et al., 1985). With the use of an intravital microscopy assay it was observed that fluorescein-labeled neutrophils from a LAD I boy rolled normally on inflamed rabbit venules, suggesting that they normally initiated adhesive interactions with inflamed endothelial cells (von Andrian et al., 1993). However, these cells failed to undergo activation-dependent, β_2-integrin–mediated adhesion and did

not stick, and therefore could not emigrate when challenged with a chemotactic stimulus (Fig. 53.1).

Patients with LAD I exhibit mild to moderate neutrophilia in the absence of overt infection. Marked granulocytosis with neutrophil counts in peripheral blood reaching levels of up to 100,000/μL occurs during acute infections. Defects in other myeloid functions are also observed. Binding and phagocytosis of iC3b-opsonized particles are also deficient, in agreement with the function of Mac-1 as complement receptor 3 (CR3) (Le Cabec et al., 2002). Abnormalities of neutrophil-mediated, antibody-dependent cellular cytotoxicity have been also reported in several patients (Kohl et al., 1986).

A wide variety of in vitro abnormalities of lymphocyte function have also been described in LAD I, but their in vivo relevance remains unclear. Although no humoral immune deficiency has been described in most of the patients, an impaired primary and secondary antibody response to a T-dependent neoantigen was reported (Ochs et al., 1993).

Molecular Basis

The CD11/CD18 integrin receptor is expressed on all leukocytes and plays an important role in multiple leukocyte functions. However, the predominant clinical manifestation of LAD I—markedly impaired phagocyte emigration in response to pyogenic infections—can be attributed to reduced neutrophil adhesion and impaired transendothelial migration due to the lack of CD18 expression by patient neutrophils.

Early on, several lines of evidence supported an autosomal recessive pattern of inheritance. Equal numbers of male and female patients were described, and family studies showed heterozygous male and female carriers who expressed 50 percent of the normal amount of the β_2-integrin molecules on their neutrophils. Furthermore, a frequent history of consanguineous marriages strongly supported the concept that LAD I is inherited as an autosomal trait (Anderson & Smith, 2001). The β_2-integrin subunit is encoded by a gene located at the distal end of the long arm of chromosome 21q22.3 (Solomon et al., 1988). In contrast to normal B-cell lines, which synthesize α_L and β_2 precursors that associate in the cytoplasm and then are transported to the cell surface as an $\alpha_L\beta_2$ heterodimer, lymphoblasts of LAD I patients synthesize a normal α_L-subunit precursor, which is never expressed on the cell surface and is apparently degraded in the absence of a normal β_2 subunit (Springer et al., 1984).

Heterogeneity among LAD I patients with respect to the extent of β_2-integrin deficiency on the leukocyte surface was first demonstrated by flow cytometry. As previously discussed, the severity of the clinical features and the magnitude of the functional deficits observed were directly related to the degree of CD18 deficiency, but the underlying molecular basis for this heterogeneity remained largely undefined until the β_2-subunit gene (*ITGB2*) was cloned and the relevant protein quantified (Anderson & Smith, 2001).

Several LAD I variants were subsequently reported to share a defect in β_2-integrin adhesive function despite normal surface expression of CD18. A child with classical LAD I features with normal surface expression of CD18 was reported (Hogg et al., 1999), in whom a mutation in CD18 led to a nonfunctional molecule. Another child with a moderately severe form of LAD I was found to have a novel point mutation in CD18, resulting in the expression of dysfunctional β_2 integrin (Mathew et al., 2000).

Mutation Analysis

The molecular basis for CD18 deficiency varies (Vihinen et al., 2001). In some cases the mutation causes diminished expression of CD18 mRNA. In other families there is expression of mRNA or protein precursors of aberrant size with either larger or smaller CD18 subunits. In other cases LAD I results from a failure to process normal-sized protein precursors to the mature normal product (Wright et al., 1995). Analysis at the gene level has revealed a degree of heterogeneity, which reflects this diversity. A number of point mutations have been reported, some of which lead to the biosynthesis of defective proteins with single amino acid substitutions, while others lead to splicing defects, resulting in the production of truncated and unstable proteins. In one case a 90-nucleotide deletion in the CD18 mRNA produced an in-frame deletion of a 30-amino acid region. Analysis of the genomic DNA showed this 90 bp region to be encoded by a single exon (exon 9). The 30-amino acid deletion and many additional mutations are located in a region encompassing residue 241 of the CD18 molecule that is highly conserved (Sligh et al., 1992) (Fig. 53.3). Domains within this segment are presumably required for biosynthesis of the subunit precursors and may contain critical contact sites between the α and β subunits.

Several splicing defects resulting in the production of abnormal β_2 protein products were also reported. Other mutations were found in exons 7 and 13 (Anderson & Smith, 2001). Although in most cases point mutations or small insertions or deletions in the CD18 (*ITGB2*) gene have been reported, an infant with LAD I suffered from a gross abnormality in chromosome 21 due to a deletion of q22.1–3 (Rivera-Matos et al., 1995).

Homozygosity for the *CD18* gene mutation was described in some cases, whereas in other cases compound heterozygosity was observed. So far over 20 different β_2 mutations have been identified to result in the LAD I phenotype (Fig. 53.3), but since in many cases of LAD I no molecular analysis has been performed, additional β_2 mutations may exist.

Strategy for Diagnosis

In any infant, male or female, with recurrent soft tissue infections and a very high leukocyte count, the diagnosis of LAD I should be considered. The diagnosis is even more suggestive if a history of delayed separation of the umbilical cord is present. To confirm the diagnosis, absence of the α and β subunits of the β_2-integrin complex has to be demonstrated. This can be accomplished with the use of appropriate anti-CD11 and -CD18 monoclonal antibodies and flow cytometry or by sequence analysis to define the exact molecular defect in the β_2 subunit. In rare cases, when β_2 integrins are normally expressed, functional in vitro assays must be performed to diagnose LAD I variants.

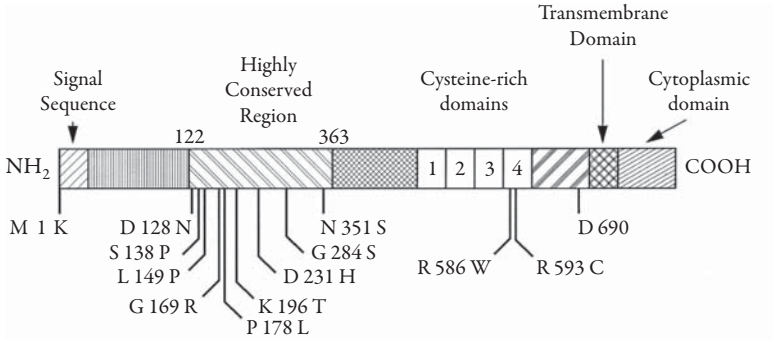

Figure 53.3 Representative mutations in the β_2-integrin subunit that have been identified in patients with LAD I syndrome.

Carrier Detection and Prenatal Diagnosis

LAD I carriers have 40 to 60 percent of normal CD18 and lack clinical symptoms. Sequence analysis shows one normal and one abnormal gene. Because leukocytes express CD18 on their surface only at and after 20 weeks of gestation, cordocentesis performed at this age can establish prenatal diagnosis (Weening et al., 1991). In families in whom the exact molecular defect has been previously identified, an earlier prenatal diagnosis is possible by chorionic biopsy and mutation analysis.

Treatment and Prognosis

Patients with the moderate LAD I phenotype usually respond to conservative therapy and the prompt use of antibiotics during acute infectious episodes. Prophylactic antibiotics may reduce the risk of infections in these patients.

Transfusion of granulocytes immediately after harvest by leukapheresis has been found to be useful in selected cases of infections that otherwise could not be controlled. Although granulocyte transfusions may be life-saving, their use is limited because of difficulties in supply of daily donors and immune reactions to the allogeneic leukocytes.

At present, the only corrective treatment that should be offered to all patients with the severe phenotype is bone marrow transplantation (Thomas et al., 1995). A recent multicenter study showed that the overall success rate in 36 patients with LAD I was 75 percent. Transplanted patients with fully matched HLA donors had a much better prognosis than those who underwent haploidentical transplantation (Qasim et al., 2009).

The introduction of a normal β_2-subunit gene (*ITGB2*) into hematopoietic stem cells has the potential to cure children with LAD I (Bauer & Hickstein, 2000). Retroviral-mediated transduction of the CD18 gene was shown to reconstitute a functional CD11a/CD18 in lymphoblastoid cell lines derived from patients with LAD I, and transduction of murine bone marrow cells with CD18 retroviral vector resulted in gene expression in a variable percentage of neutrophils at 2 weeks posttransduction (Wilson et al., 1993). Retroviral-mediated transfer of the *CD18* gene into primary LAD I bone marrow cells has also been reported (Bauer et al., 1998). Expression of CD18 was observed in a low percentage of neutrophils in two LAD I patients undergoing gene therapy without conditioning (Bauer et al., 1998). Successful LAD gene therapy with foamy viral vectors was recently reported in a canine model (Bauer et al., 2008). Taken together these studies give hope that gene therapy can be used for treatment of LAD I whenever bone marrow transplantation is unavailable.

Recently, a new approach to treat LAD I has been tried (Simon et al., 2010). As in cystic fibrosis, the use of aminoglycoside antibiotics (e.g., gentamicin) in vivo as in vitro can partially correct the premature protein termination by induction of a read-through mechanism. This approach increased the expression of CD18 but, unfortunately, did not improve leukocyte adhesion in two such treated LAD I patients.

CD18-Deficient Mice

Multiple mouse models with targeted disruption of one or more leukocyte and endothelial adhesion molecules have been generated and explored using multiple inflammatory model systems as well as intravital microscopy. Of particular interest are those knockouts that most closely mimic individual LAD syndromes both phenotypically and genetically.

Mutant mice with complete deficiency of CD18 resemble phenotypically the LAD I syndrome (Scharffetter-Kochanek et al., 1998). As in LAD I, these animals exhibit marked neutrophilia (11- to 30-fold increase over wild type). The granulocytosis observed in the CD18-deficient mice was due mainly to continuous stimulation of the bone marrow and enhanced production of myeloid cells as a consequence of the defective antimicrobial functions leading to infections (Horwitz et al., 2001). Similar to LAD I, there was almost no emigration of neutrophils in the CD18-null animals into sites of inflammation involving the skin. In contrast, the CD18-deficient mice exhibited comparable numbers of neutrophils in lavage fluid from inflamed peritoneum and increased emigration into inflamed lung when compared with wild type. The persistence of neutrophil emigration into the inflamed peritoneum in the CD18-null animals may result from the marked increase in circulating neutrophils in the CD18-deficient animals. Because the number of emigrating neutrophils is in part dependent on the number of circulating neutrophils, similar absolute numbers of neutrophils may have eventually emigrated in the CD18 null animals, although with markedly reduced rates.

CD18-Deficient Dogs and Cattle

A spontaneous mutation in the gene coding for the β_2 single integrin has been described in both dogs (Giger et al., 1987) and cattle (Shuster et al., 1992). Canine LAD (CLAD) occurs in Irish setters and results from a single missense mutation in the *CD18* gene, resulting in a substitution of cysteine to serine at position 36 (Kijas et al., 1999). In bovine LAD (BLAD) there is a also a point mutation (Asp128Gly) in the CD18 gene. Animals homozygous for this mutation exhibit persistent neutrophilia and suffer from recurrent infections associated with poor growth performance. Interestingly, the carrier frequency of the defective gene among Holstein cattle has been approximately 15 percent in bulls and 6 percent in cows (Shuster et al., 1992).

LAD II

LAD II (OMIM266265) is caused by a rare defect in fucose metabolism, resulting in the absence of SLeX and other structurally related fucosylated selectin ligands. LAD II was first described in two unrelated Palestinian boys, each the offspring of consanguineous parents (Etzioni et al., 1992). Subsequently, two additional unrelated Palestinian children and one Turkish child (Etzioni & Tonetti, 2000), a child of Brazilian origin (Hidalgo et al., 2003), and one from Pakistan (Yakubenia & Wild, 2006) have been reported, reaching a total of six reported LAD II patients.

Clinical and Pathological Manifestations

Affected children are born after uneventful pregnancies with normal height and weight. No delay in the separation of the umbilical cord has been observed. Patients with LAD II have severe mental retardation, short stature, a distinctive facial appearance, and the rare Bombay blood phenotype. They develop recurrent episodes of bacterial infections early in life, mainly pneumonia, periodontitis, otitis media, and localized cellulitis. Mild to moderate skin infections without obvious pus have also been observed (Table 53.2). The infections are generally not life-threatening and can usually be treated in the outpatient clinic (Etzioni et al., 1998). Interestingly, after the age of 3 years, the frequency of infections decreases and the children no longer need prophylactic antibiotics.

At an older age, the main infectious problem of LAD II patients is severe periodontitis, also observed in patients with LAD I. During times of infections the neutrophil count increases up to 150,000/μL. It is of interest to note that from their first days of life LAD II patients exhibit neutrophilia, ranging between 25,000 and 30,000/μL, even when they are free of infections.

Overall, the infections in LAD II appear to be comparable to the moderate rather than the severe phenotype of LAD I. It is possible that the ability of LAD II neutrophils to adhere and transmigrate via β_2 integrin under conditions of reduced shear forces (von Andrian et al., 1993) may permit some neutrophils to emigrate to sites of severe inflammation where blood flow is low due to vasodilatation. Thus, some level of neutrophil defense against bacterial infections can be obtained in LAD II patients but not in patients with severe LAD I.

As in LAD I, no pus formation at the site of infection occurs, and there is a striking absence of neutrophils in the lesions. Lymphocytic infiltration, by contrast, is clearly present (see below).

The severe mental retardation is reminiscent of congenital disorders in glycosylation (CDGs), a heterogeneous group of autosomal recessive disorders with defects in the processing and synthesis of carbohydrate moieties of multiple glycoproteins (Jaeken & Matthijs, 2007). While in early infancy the immune defect due to leukocyte adhesion deficiency is the main clinical problem, later on the general metabolic consequences of the defect in fucose metabolism govern the clinical picture. This syndrome should be therefore designated as LAD II/CDG IIc.

Laboratory Findings

The clinical picture of skin infections, pneumonia, and periodontitis associated with a very high blood neutrophil count initially suggested a neutrophil defect as the cause of this syndrome. Subsequently it was found that although the opsonophagocytic activity was normal, a marked defect in neutrophil motility was shared by all LAD II patients (Etzioni et al., 1992). Both random migration and directed migration toward chemotactic factors were markedly decreased (10 percent of normal) despite the independence of these assays on selectin–carbohydrate interactions.

Since homotypic aggregation of patient neutrophils, a process initiated by L-selectin–dependent adhesions (King et al., 2005), was found to be defective (Etzioni, 2009), these observations suggested that the problem was a defect in neutrophil adherence to surfaces; therefore, the possibility of a defect in adhesion molecules was investigated. Indeed, while patients' and their parents' neutrophils exhibited normal levels of integrin subunits, LAD II neutrophils were found to be deficient in expression of the SLeX antigen (Etzioni et al., 1992). In contrast to LAD I neutrophils, which failed to flatten and spread on phorbol ester-treated glass coverslips, the majority of LAD II neutrophils flattened and spread extensively, consistent with normal integrin functions (Phillips et al., 1995). Nevertheless, a small percentage of the LAD II cells exhibited an intermediate appearance and retained a more spherical shape. The poor migration toward chemoattractants in an under-agarose assay and the defective homotypic neutrophil adhesion could not be readily explained by the biochemical deficiency of SLeX and suggested a more global defect in cell activation or adhesion, possibly due to aberrant L-selectin–mediated signaling. Nevertheless, β2-integrin upregulation in LAD II neutrophils was normally induced by multiple agonists that bypass L-selectin signaling (Phillips et al., 1995).

Functional Aspects

To examine further the significance of the fucosylation defect, both in vitro and in vivo functions of LAD II

neutrophils were assessed. Neutrophils isolated from the peripheral blood of one LAD II patient failed to bind to purified platelet-derived P-selectin and recombinant E-selectin in vitro (Phillips et al., 1995). To determine the effect of SLeX deficiency on neutrophil–endothelial interactions, neutrophil adherence to IL-1-, TNF-, histamine-, and phorbol ester-activated human umbilical vein endothelial cells was examined. No adhesion of LAD II neutrophils was observed when the endothelial cells were activated with IL-1β (Fig. 53.4) or TNF-α, both potent inducers of E-selectin, whereas normal neutrophils bound avidly to these stimulated endothelial cells. Similarly, endothelial cells activated by histamine to express P-selectin bound normal neutrophils but not LAD II neutrophils. By contrast, normal neutrophil adhesion to endothelial cells was observed after activation of the LAD II leukocytes with phorbol myristate acetate (PMA), an activating stimulus for β_2 integrins (Fig. 53.4). Furthermore, LAD II neutrophil migration across endothelial monolayers in response to f-Met-Leu-Phe (fMLP), a CD18-dependent function, was comparable to that of normal neutrophils (Phillips et al., 1995).

Rolling, the first step in neutrophil recruitment to the site of inflammation, is mediated primarily by the binding of the selectins to their fucosylated glycoconjugate ligands. Through intravital microscopy, the in vivo behavior of fluorescein-labeled neutrophils from a normal donor and from LAD I and LAD II patients was investigated during their passage through the inflamed microcirculation of rabbit mesentery (von Andrian et al., 1993). The rolling fraction of normal donor neutrophils in this assay was approximately 30 percent, and LAD I neutrophils behaved similarly. In contrast, LAD II neutrophils rolled poorly and failed to emigrate (Fig. 53.1). Their rolling fraction was only 5 percent (Fig. 53.5), and most of the cells that did interact with endothelial cells had a higher rolling velocity, rolled only over short distances, and frequently detached from the vessel wall. This marked inability to interact with inflamed vasculature was observed only in the presence of intravascular shear force: when the mesenteric blood flow was temporarily stopped and LAD II cells were injected into the nonperfused microvasculature, numerous LAD II cells became stuck and did not detach when the blood flow was subsequently restored. These experiments indicate that the defect in LAD II is due to a shear-dependent inability of neutrophils to roll and slow down in inflamed venules and is not due to a dysfunction of the later integrin-mediated steps in the adhesion cascade.

To examine in vivo extravasation, the response of patient neutrophils to cutaneous inflammation was assessed by both skin-chamber and skin-window techniques. Neutrophil emigration was markedly diminished in both tests, the values being approximately 1.5 percent and 6 percent of normal in the skin-window and skin-chamber tests, respectively (Price et al., 1994). Monocyte migration to the skin-window site was reduced to a similar degree. Notably, neutrophils from a patient with LAD I, studied concurrently, showed the same magnitude of defect in these assays. Nevertheless, as only a residual amount of LAD leukocytes could establish firm adhesions on inflamed vessels, the lack of extravasation could not be attributed to defects in the actual diapedesis/endothelial crossing steps or to chemotactic activity of LAD cells.

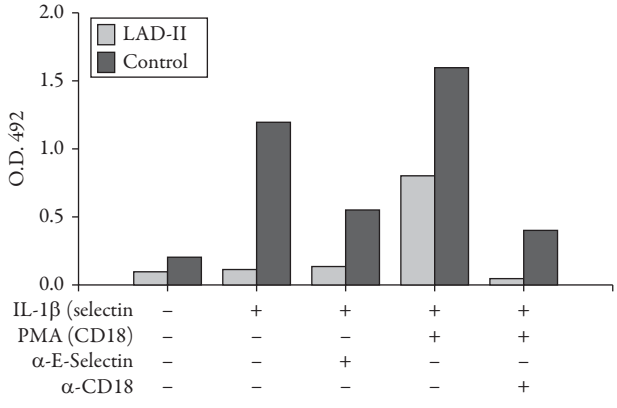

Figure 53.4 LAD II neutrophil adhesion to endothelial cells. Selectin-dependent adhesion of LAD II neutrophils in vitro is impaired in LAD II syndrome. Purified neutrophils from a normal donor (*light shaded columns*) or the LAD II patient (*dark columns*) were incubated for 5 minutes on human umbilical vein endothelial cell monolayers that had been treated with control medium or with recombinant human IL-1. In some wells neutrophil CD18 was activated by addition of PMA. An anti-CD18 monoclonal antibody or an anti–E-selectin monoclonal antibody was added to some wells during incubation. The plates were washed and neutrophil adherence was quantitated by spectrophotometric analysis of the neutrophil-specific enzyme myeloperoxidase (Etzioni et al., 1992; Phillips et al., 1995).

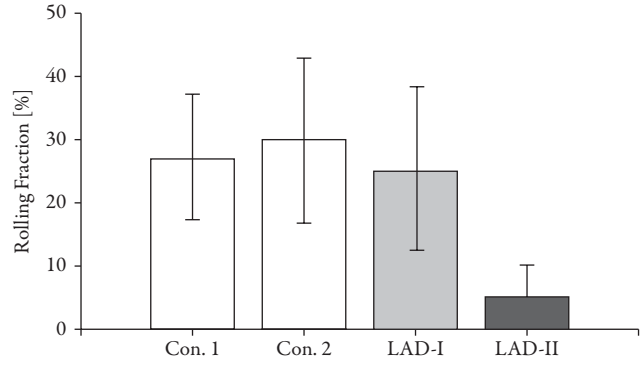

Figure 53.5 LAD I and LAD II neutrophil rolling in inflamed rabbit mesenteric vessel. Selectin-dependent rolling of neutrophils in vivo is reduced in LAD II syndrome. Neutrophil rolling from control subjects and patients with LAD I and LAD II was assessed quantitatively in inflamed mesenteric venules of rabbits. Samples of fluorescent neutrophils were injected into the terminal mesentery artery blood stream 4 to 6 hours after intraperitoneal injection of IL-1. Cells passing through venules in the downstream segment were made visible by stroboscopic epi-illumination and were recorded on videotape. Tapes were analyzed for assessment of neutrophil rolling behavior. The rolling fraction was determined as the percentage of rolling neutrophils in the total flux of fluorescent cells passing a venule during an injection (Arbones et al., 1994). A total of 10 venules was analyzed, 5 vessels for each neutrophil sample in each rabbit. Means ± SD are shown (von Andrian et al., 1993). (Reproduced from the *Journal of Clinical Investigation* 1993;191:2893–2897, by copyright permission of the American Society for Clinical Investigation.)

Adhesion molecules are known to participate in the immune reaction of T lymphocytes (Springer, 1995), and, as noted above, some lymphocyte functions are defective in LAD I (Ochs et al., 1993). In contrast, LAD II patients show a normal proportion and absolute numbers of T-lymphocyte subpopulations. They also exhibit normal proliferative responses to various mitogens, normal NK-cell activity, and normal immunoglobulin levels. Furthermore, in contrast to patients with LAD I, antibody production in response to immunization with bacteriophage 0X174 was found to be normal, with appropriate isotype switching from IgM to IgG (Price et al., 1994). However, delayed cutaneous hypersensitivity reactions were not observed upon intradermal injection of various antigens. This apparent defect may be due to absence of the skin homing T-cell antigen, CLA (Picker, 1994), a fucose-containing glycan related to the SLeX carbohydrate motif lacking in LAD II neutrophils. Therefore, the in vivo immune response to keyhole limpet hemocyanin (KLH) was investigated. Normal KLH-specific in vitro T-cell proliferation and normal in vivo anti-KLH antibody titers were found, indicating normal T- and B-cell function. However, skin testing with KLH failed to elicit a positive reaction (rubor, calor, edema) (Kuijpers et al., 1997a). A biopsy of the site of intradermal KLH injection revealed that the number and phenotype ($CD3^+$) of infiltrating lymphocytes were identical to those of a positive control (except for the lack of CLA staining in the LAD II biopsy). Upregulation of various endothelial adhesion molecules was also similar in the patient and control. The discrepancy between the almost normal histological findings and the absence of an intradermal reaction could be explained by reduced skin homing of subsets of T cells. These T-cell subsets may prime the skin-associated inflammatory reaction and initiate the classical signs of delayed-type hypersensitivity reaction in the skin, but their contribution to inflammatory processes in other tissues is minimal.

Molecular Basis

Because the first two LAD II patients identified were the offspring of first-degree relatives and the parents were clinically unaffected, autosomal recessive inheritance was assumed (Etzioni et al., 1992). In addition to being of the Bombay phenotype (absence of the fucose-containing H antigen), the cells of LAD II patients were found to lack Lewis a and b antigens. Therefore, it was concluded that the biochemical abnormality in LAD II was a general defect in fucosylation of macromolecules (Fig. 53.6). Because at least four different fucosyltransferases would have to be affected to produce complete negativity for SLeX, H, Lewis, and secretor glycoconjugates and the genes in these molecules are not physically linked in the human genome (loci include chromosomes 11, 19p, and 19q), LAD II cannot result from abnormal fucosyltransferase(s). Furthermore, LAD II patients possess normal activity of α-2-fucosyltransferase (Shechter et al., 1995). This transferase is required for H antigen biosynthesis and is defective in individuals who carry the rare Bombay blood phenotype but do not have LAD II. These findings suggest that the primary defect in LAD II must be a general defect in fucose production rather than fucose transferases.

Although the defect in the Palestinian patients was suggested to involve impaired de novo GDP-1-fucose biosynthesis (Karsan et al., 1998), the two enzymes involved in this pathway, GMD and FX protein, were found to be normal, without a mutation in cDNA isolated from LAD II patients. Subsequently, the Turkish child with LAD II was found to have decreased GDP-1-fucose transport into the Golgi vesicles (Lubke et al., 1999). Similar studies were performed in the Palestinian patients and, indeed, the same general defect in fucose transport to the Golgi vesicles was found. Still, marked kinetic differences were observed between the Turkish and the Palestinian patients (Sturla et al., 2001). These findings may explain the different response to fucose supplementation in the Turkish and the Palestinian children (see below). Subsequently, through use of the complementation cloning technique, the human gene encoding the fucose transporter was found to be located on chromosome 11 (Lubke et al., 2001). The Turkish child was homozygous for a mutation at amino acid 147, in which arginine is replaced by cysteine (Lubke et al., 2001; Luhn et al., 2001), whereas the two Palestinian patients examined had a homozygous mutation in amino acid

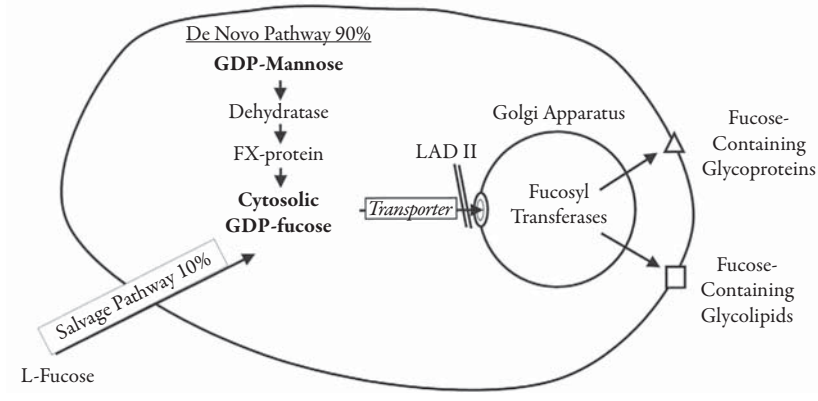

Figure 53.6 LAD II is a congenital disorder of glycosylation. GDP-l-fucose is synthesized in the cytosol and must be transported into the Golgi apparatus where fucosyl transferases are located. In the Golgi, fucosyl transferases add fucose to macromolecules. In LAD II, deficiency of the GDP-fucose transporter prevents fucosylation of membrane glycoproteins and glycolipids.

308, in which threonine is changed to arginine (Lubke et al., 2001). Both mutations are located in highly conserved transmembrane domains of the fucose transporter. The Brazilian patient identified in 2003 has a homozygous single nucleotide deletion (588delG) resulting in frameshift and premature stop codon 34 residuals after the mutation (Hidalgo et al., 2003). LAD II is thus one of the CDGs and is classified as CDG IIc (Lubke et al., 2001). Although only three unique mutations (in five affected patients) have been described thus far, there seems to be a genotype–phenotype correlation (Etzioni et al., 2002). Recently it was observed that some mutations allowed nonfunctional transporter to be present in the Golgi apparatus, while others prevented localization of the transporter in the Golgi (Helmus et al., 2006).

Strategies for Diagnosis

LAD II is a very rare syndrome, but because the clinical phenotype is striking, the diagnosis can be made based on the presence of recurrent, albeit mild, infections, marked leukocytosis, and the Bombay blood group, in association with mental and growth retardation.

Analysis of peripheral blood leukocytes by flow cytometry with an anti-CD15s monoclonal antibody that recognizes the SLeX carbohydrate can readily demonstrate lack of this carbohydrate on patient leukocytes. To confirm the diagnosis, sequence analysis of the gene encoding the GDP-fucose transporter is required.

Carrier Detection and Prenatal Diagnosis

Prenatal diagnosis was carried out successfully through use of a cord blood sample obtained from a female fetus of one of the affected families. Because of the presence of the Bombay blood phenotype, the pregnancy was aborted (Frydman et al., 1996). Once the gene causing LAD II had been cloned, carrier detection became possible, and prenatal diagnosis can be performed earlier with chorionic villus samples for DNA analysis.

Treatment and Prognosis

Each of the LAD II patients described to date suffered from recurrent episodes of infections that responded well to antibiotics. No serious consequences were observed, and prophylactic treatment was never considered in the Palestinian patients, whereas the Turkish child was placed on prophylactic antibiotics. A chronic problem for all patients has been periodontitis, a condition that is especially difficult to treat in children with severe mental retardation (Etzioni et al., 1998). The oldest LAD II patient is now 22 years old and has severe psychomotor retardation, but only mild infectious problems after removal of his teeth.

Because of the proposed defect in fucose production, supplemental administration of fucose through either addition to the diet or intravenous infusion has been suggested. Indeed, fucose supplementation caused dramatic improvement in the condition of the Turkish child (Marquardt et al., 1999). A marked decrease in leukocyte count with improved neutrophil adhesion to endothelial cells was noted in vitro. Furthermore, improvement in developmental delay was observed and no episodes of infections occurred during fucose therapy. Despite this success, the mental retardation remained untreatable.

Notably, the same protocol produced no improvement in laboratory data or clinical symptoms in two Palestinian children (Etzioni & Tonetti, 2000). This difference may be due to the genetic defect in the Turkish child, which results in decreased affinity of the transporter for fucose. An increase in cytosolic concentration of fucose would be expected to overcome, at least in part, the defect in fucose transport.

Selectin- and Selectin Ligand-Deficient Mice

Although the genetic deficiency in LAD II has no direct effect on selectin genes, the resulting defect in fucose metabolism produces a deficiency of fucosylated ligands for endothelial E- and P-selectins. The endothelium in LAD II patients, especially lymph node HEVs and chronically stimulated postcapillary venules likely, also exhibits reduced expression of fucosylated leukocyte L-selectin ligands (Karsan et al., 1998). Thus, with respect to leukocyte adhesion, LAD II patients may suffer from similar defects as mice with combined knockout of E-, P-, and L-selectin genes.

Mice deficient in E- and P-selectin (E/P) and mice deficient in E-, P-, and L- selectin (E/P/L) indeed show leukocytosis, dramatic reduction in leukocyte rolling, markedly reduced emigration into inflamed peritoneum, and increased susceptibility to mucocutaneous infections (Bullard et al., 1996; Frenette et al., 1996; Jung & Ley, 1999; Robinson et al., 1999). In contrast to the E/P- or E/P/L-deficient mice, the two older LAD II patients have had no increase in systemic infections, and the few episodes of localized infection have responded to conventional treatment as in any immunocompetent child. The phenotype of the multiple selectin-deficient animals thus appears to be much more severe than that observed in LAD II, perhaps reflecting differences between humans and mice in susceptibility to mucocutaneous infections. However, the possibility that nonfucosylated ligands participate in selectin interactions in vivo in humans more than in mice has not been completely excluded. A better mimic of LAD II would be mice deficient in biosynthesis of SLeX. The gene encoding $\alpha(1,3)$ fucosyltransferase Fuc-TVII, an enzyme that controls synthesis of SleX-containing glycans both in leukocytes and HEVs, was knocked out in mice, resulting in a phenotype reminiscent of the human LAD II, with defective adhesion and marked leukocytosis (Maly et al., 1996). The same phenotype was observed in mice deficient in core 2 $\beta 1$–6 *N*-acetylglucosaminyl transferase, another critical factor in the synthesis of SLeX (Ellies et al., 1998). However, neither murine model showed any growth abnormalities, confirming that the growth and mental retardation in LAD II is most likely due to the general defect in fucose metabolism and not to the adhesion deficiency.

Golgi GDP-1-Fucose Transport)–Deficient Mice

To investigate further the role of the Golgi GDP fucose transporter, knockout mice were generated (Sic35c1$^{-/-}$ mice). Such mice displayed severe growth retardation, an elevated postnatal mortality rate, and severe impairment of P-, E-, and L-selectin–mediated rolling functions in multiple types of leukocytes (Hellbusch et al., 2007). Furthermore, a tremendous reduction of fucosylated glycoconjugants in many organs and isolated cells was observed. In a follow-up report the Sic35c1$^{-/-}$ mice demonstrated a defect in rolling that was accompanied by strong but not completely abolished leukocyte adhesion. Although strong defective leukocyte trafficking was observed, normal lymphocyte homing to the spleen was noted (Yakubenia et al., 2008), which may explain the largely normal lymphocyte function in LAD II.

LAD III

More recently, a rare, autosomal recessive LAD syndrome distinct from LAD I and LAD II has been reported in multiple patients of Turkish, Palestinian, and Maltese origin (Kuijpers et al., 1997b; McDowall et al., 2003). All patients had similar clinical symptoms, characterized by severe recurrent infections, a bleeding tendency, and marked leukocytosis (Table 53.2).

Some of these unique patients were considered as LAD I variants (Kuijpers et al., 1997b; McDowall et al., 2003) due to the severely impaired β_2-integrin functions associated with this syndrome. Because LAD I leukocytes have defects in integrin expression or structure, it was proposed that this group of integrin-activation disorders be designated *LAD III* (Alon & Etzioni, 2003; Kinashi et al., 2004).

Although leukocyte integrin expression has been found to be normal in LAD III (OMIM612840), in situ activation of all major leukocyte integrins, including LFA-1, Mac-1, and VLA-4, by chemokines or chemoattractants is severely impaired in patient-derived lymphocytes and neutrophils. In addition, a marked defect in platelet aggregation was described and demonstrated to be associated with inability of the major platelet integrin, GpIIbb3, to undergo activation by multiple agonists (Kuijpers et al., 1997b). Whereas the rolling of LAD III leukocytes on endothelial surfaces is normal, lymphocytes fail to arrest on endothelial integrin ligands in response to endothelial-displayed chemokines, while neutrophils fail to arrest on integrin ligands after short rolling on E-selectin (Fig 53.7) (Alon et al., 2003). G-protein–coupled receptor (GPCR) signaling appears to be normal, and the ability of leukocytes to migrate toward a chemotactic gradient is not impaired. The key defect in this syndrome was originally attributed to a genetic loss of integrin activation by GPCR signaling (Alon et al., 2003). Nevertheless, in all of the LAD III cases studied, variable defects in activation of leukocyte integrins by non–GPCR-mediated signals, such as phorbol esters and integrin ligands, were observed (Kuijpers et al., 1997b; McDowall et al., 2003). Furthermore, cell lines derived from patient lymphocytes failed to spread on isolated integrin ligands even in the absence of exogenous agonists (Malinin et al., 2009; Svensson et al., 2009).

Although integrins are expressed normally on all patient cells, neither β_2 or β_3 integrins can undergo inside-out conformational activation, and both Mac-1 and GpIIbIIIa fail to bind soluble ligands in response to potent agonists (Malinin et al., 2009; Pasvolsky et al., 2007). As these processes were shown to require a variety of GTPases, the LAD III phenotype was predicted to involve the loss of a GTPase or of one of its activating guanine nucleotide exchange factors (GEF) in both leukocytes and platelets of LAD III patients.

Molecular Basis

While for several years the primary genetic defect was elusive, recent studies of integrin-activation defects in knockout mouse models revealed the molecular basis of the disease. A

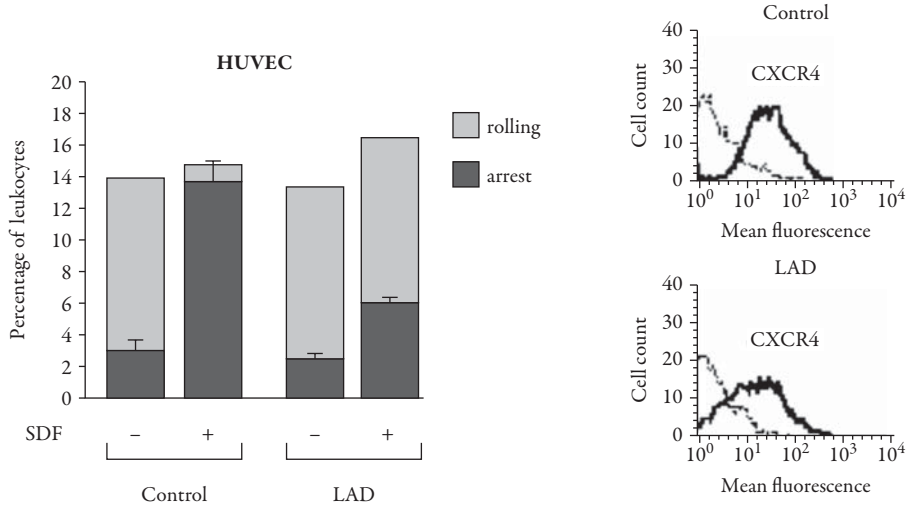

Figure 53.7 Defective adhesion of lymphocytes to human umbilical vein endothelial cells (HUVECs) after stimulation with chemokine in LAD III. While the rolling of lymphocytes is normal in LAD III, a marked decrease in lymphocyte adhesion after stimulation with chemokine (SDF1) can be observed (left panel), although chemokine ligand (CXCR4) is expressed normally (right panel).

critical component of integrin activation by GPCR signals is the GTPase Rap1. Although Rap1 expression is widespread in all tissues, "inside-out" activation of integrins triggered by GPCR signals was recently linked to a novel Rap1 GEF, CalDAG-GEF-1, which is expressed mainly in platelets, leukocytes, and brain. This GEF catalyzes the exchange of GTP for GDP bound to Rap1 and is crucial for GPCR-mediated integrin activation in platelets and neutrophils (Crittenden et al., 2004). A mouse knockout model exhibited the same defects in β_1-, β_2-, and β_3-integrin activation in both platelets and leukocytes, suggesting that this mouse model represents a model for the molecular defect in LAD III (Bergmeier et al., 2007). Indeed, a large group of LAD III patients, all from Turkish origin, exhibit a splice junction mutation in their *CalDAG-GEF-1* (official symbol *RASGRP2*) gene with nearly complete loss of messenger RNA and protein expression in patient platelets, neutrophils, and primary T lymphocytes (Pasvolsky et al., 2007). Nevertheless, other patients of non-Turkish origin were found to express normal CalDAG-GEF-1, and cell lines derived from these patients exhibited intact Rap1 activation (Malinin et al., 2009). Since these cell lines exhibited defective spreading on isolated integrin ligands as well as defects in integrin activation by non-GPCR agonists, the LAD III phenotype of these patients could represent a broader defect in integrin conformation downstream of chemokine receptors and GPCR-triggered GTPases. The cytoplasmatic tails of the different integrins affected in LAD cells are rather short but are recognized by various adaptor proteins, which bind and promote integrin affinity to their ligands either by inside-out signals or by being occupied by integrin ligands that induce extensive conformational changes of the extracellular domain of the integrin, a process termed outside-in integrin activation (Hynes, 2002). One such key adaptor with broad activities is talin, shown to activate integrin affinity and adhesiveness in many cellular systems (Moser et al., 2009b).

Talin 1, the predominant talin expressed in hematopoietic cells, plays a critical role in integrin activation in all cell types, and its deletion in mice is embryonically lethal (Legate & Fassler, 2009); however, talin deficiency was ruled out in LAD III patients.

A breakthrough in the search for the molecular defect causing LAD III was achieved when a family of integrin cytoplasmic tail binding adaptors, termed Kindlins, were identified as being of crucial importance for maximal integrin affinity regulation of talin (Ma et al., 2008). One of these three family members, Kindlin-3, is nearly exclusively expressed on hematopoietic cells (Ussar et al., 2006). Kindlin-3 (OMIM607901) is located on chromosome 11q12 and has a molecular weight of 75.9 KD. It consists of 667 amino acids and 15 exons. Platelet aggregation and GPCR-triggered integrin activation were diminished in kindlin-3 knockout mice (Moser et al., 2008). A subsequent study on neutrophils and monocytes derived from these kindlin-3 knockout mice revealed total loss of β2 integrin adhesiveness both in vivo and in vitro as well as defective activation of the monocyte β_1 integrin, and VLA-4 by chemokine signals (Moser et al., 2009a). Thus, kindlin-3 knockout mice represented a genetic model of a LAD III-like phenotype. Furthermore, Kindlin-3 was found to be also involved in osteoclast-mediated bone resorption (Schmidt et al., 2011). Interestingly, some LAD III patients develop osteopetrosis-like features (Kilic & Etzioni, 2009; Malinin et al., 2009; Sabnis et al., 2010). Since, unlike talin, Kindlin-3 expression is restricted to platelets, leukocytes, and red blood cells, the global integrin deficiency in β_3, β_2, and β_1 integrin members expressed by these multiple cell types in mice suggested that Kindlin-3 deficiency, on its own, could be the cause of LAD III in humans expressing intact CalDAG-GEF-1.

Indeed, mutation analysis of Kindlin-3 (official symbol FERMT3) revealed homozygous mutations in most LAD-III patients regardless of their origin (Table 53.3). Sequencing of the *Kindlin-3* gene revealed a homozygous nonsense mutation (R513X) in three Turkish patients that carry also a CalDAG-GEF-1 mutation (Mory et al., 2008). Kindlin-3 and CalDAG-GEF-1 are both located at 11q12–13, although the two genes are 503,029 bp apart, suggesting that both mutations were inherited as a common allele in these patients. Subsequent studies (Kuijpers et al., 2009; Malinin et al., 2009; Robert et al., 2011; Svensson et al., 2009) showed several distinct mutations in Kindlin-3 in multiple non-Turkish LAD III patients, all of Middle Eastern or Mediterranean origin. An African-

Table 53.3 **MUTATION IN KINDLIN 3 (FERMT3) (NM_031471)**

EXON/INTRON	MUTATION	PROTEIN CHANGE	TYPE	ORIGIN	REFERENCE
Exon 2	c.46G>A	W16X	nonsense	Arab (UAE)	Malinin et al., 2009
Intron 3	c.310–2A>C		splice defect	Gypsy	Robert et al., 2011
Exon 6	c.687G>A	W229X	nonsense	Arab (Israel)	Mory et al., 2008
Exon 8	c.922G>A	G308R	missense	African-American	McDowall et al., 2010
Exon 11	c.1275delT	frameshift stop	deletion		
Exon 12	c.1525C>T	R509X*	nonsense	Turkish*	Mory et al., 2008; Svensson et al., 2009
Intron 13	c.1671–2A>G		splice defect	Malta	(Svensson et al, 2009)
Exon 14	c.1717C>T	R573X	nonsense	Turkish	(Kuijpers et al, 2009)

*This mutation was found in several Turkish families, while all other mutations were described as occurring only once in a single individual family.

American patient with two novel homozygous mutations on each allele (one possibly a polymorphism) was also reported recently (Table 53.3) (McDowall et al., 2010). Regardless of the location of these mutation, all patients exhibited decreased Kindlin-3 mRNA levels and total loss of protein expression. Transfection of patient-derived lymphoblast lines with wild-type Kindlin-3 rescued the LAD III defect by restoring integrin-mediated adhesion spreading and motility (Malinin et al., 2009; McDowall et al., 2010; Svensson et al., 2009). Kindlin-3 binds to an NXXY motif on the cytoplasmic tail of the β subunit of LFA-1 (Moser et al., 2009b), facilitating the binding of the cytoskeletal protein talin to the cytoplasmic tail of the β subunit, thus initiating the formation of the integrin-activating membrane complex. Thus, Kindlin-3 selectively contributes to the TCR-triggered outside-in stabilization of bonds generated between chemokine-primed LFA-1 molecules and cell-surface ICAM-1 (Feigelson et al., 2011).

Treatment and Prognosis

Patients with LAD III suffer from similar infectious episodes as those with LAD I and thus need prophylactic antibiotics. Since in addition to β_2-integrin deficiencies, α_4 integrins on lymphocytes and monocytes are also defective, the emigration of these leukocyte subsets is more severely impaired than in LAD I, in which these integrins remain functionally intact. Due to their bleeding tendency, blood transfusions are often required; several patients died from bleeding episodes (Kuijpers et al., 2007). The only possible curative therapy of this global integrin activation deficiency is bone marrow transplantation (Elhasid et al., 2010; Sabnis et al., 2010), which corrects all clinical symptoms, including recurrent infections, bleeding tendency, and osteopetrosis.

Kindlin-3–Deficient Mice

In addition to their LAD III phenotype, mice lacking kindlin-3 suffer from fatal anemia, which is caused by excessive hemorrhage due to impaired platelet aggregation and defects in red blood cell production, possibly due to defective maturation from erythroid precursors that require kindlin-3 and integrins for its proper differentiation (Kruger et al., 2008; Moser et al., 2008). This defect is not observed in affected humans. The mice also show markedly reduced size and cellularity of spleen and thymus, lack detectable lymph nodes, and have lymphopenia, which may reflect defects in B-cell production and impaired migration of precursor T-linage cells to the lymphoid organs (Moser et al., 2009a). In contrast, in LAD III patients, T- and B-lymphocyte counts are elevated due to impaired emigration to peripheral lymphoid tissues, but lymphocyte generation from precursor cells appears normal.

OTHER LAD DISEASES

E-selectin Deficiency

Another potentially inherited defect in leukocyte trafficking resulting in deficient selectin function was described in a child with moderate neutropenia and severe recurrent infections (DeLisser et al., 1999). The syndrome is associated with markedly reduced expression of E-selectin on blood vessels of inflamed tissue with increased levels of circulating soluble E-selectin, suggesting increased endothelial cleavage of otherwise normally expressed E-selectin. Notably, the E-selectin gene sequence was normal, suggesting an abnormality in endothelial proteolytic activity, which could also affect the shedding of other proadhesive and proinflammatory endothelial surface molecules.

Rac-2 Deficiency

Two patients were reported with Rac-2 deficiency who in many aspects resemble LAD I patients, presenting with omphalitis and neutrophilia with defective neutrophil adhesion and migration. However, these patients presented with a marked defect in NADPH oxidase activity and F-actin formation, findings that distinguish them from LAD I (Pai et al., 2010).

CONCLUSIONS

The crucial role of the β_2-integrin subfamily in leukocyte emigration was convincingly demonstrated after LAD I was discovered. Patients with this disorder suffer from life-threatening bacterial infections. In its severe form, death usually occurs in early childhood unless bone marrow transplantation is performed.

The LAD II disorder clarifies the role of the selectin receptors and their fucosylated ligands such as SLeX. In vitro as well as in vivo studies establish that this family of adhesion molecules is essential for leukocyte rolling, the first step in leukocyte emigration through blood vessels. Clinically, patients with LAD II suffer from a less severe form of disease, resembling the moderate phenotype of LAD I. This may be due in part to the ability of LAD II neutrophils and lymphocytes to use integrins and other adhesion receptors to emigrate from blood vessels with low blood flow rates. The molecular defect responsible for LAD III, the most severe LAD syndrome, is due to loss of the hematopoietic integrin coactivator Kindlin-3, resulting in combined defects in leukocyte and platelet adhesion, migration, and aggregation. As in severe LAD I, death usually occurs in early childhood and bone marrow transplantation is the only curative treatment available. Prenatal diagnosis can be offered to families with a known heterozygous pregnant carrier if mutations in any of the known LAD genes have been identified.

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54.

INHERITED HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS SYNDROMES

Geneviève de Saint Basile

Several hereditary disorders of the immune system are characterized by the occurrence of a similar T lymphocyte and macrophage activation syndrome, also called hemophagocytic lymphohistiocytosis (HLH) syndrome or accelerated phase of the disease, which is generally fatal in the absence of treatment (Menasche et al., 2005). Hemophagocytic syndrome is characterized by lymphoid organ and extranodal infiltration by polyclonal, activated T cells and activated macrophages that phagocytose blood cells (Henter et al., 1991a). HLH is in most cases triggered by a viral infection, especially of the herpes group (Epstein-Barr virus [EBV] and cytomegalovirus [CMV]). Four inherited disorders have been identified that lead to HLH syndrome (Menasche et al., 2005): the familial hemophagocytic lymphohistiocytosis (FHL) (MIM 267700), the Griscelli syndrome (GS) (MIM 214450), the Chediak-Higashi syndrome (CHS) (MIM 214500), and the X-linked lymphoproliferative syndromes (MIM 308240). Recently, HLH was also reported in an unique patient with a form of Hermansky-Pudlak syndrome type 2 (Enders et al., 2006). Only autosomal recessive disease will be discussed in this chapter, the X-linked disorders being addressed in other chapters (Chapter 44). The primary causes of HLH should be distinguished from lymphohistiocytic proliferation with hemophagocytosis, which develops secondary to severe infections in an immunocompromised host, or during the course of malignancies (Janka et al., 1998).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS OF INHERITED HLH

The clinical presentation of HLH may vary widely. In nearly all patients, high fever, frequently undulant and protracted, is the first sign, in association with hepatomegaly and splenomegaly. Pallor, anorexia, vomiting, and irritability are often noted. Transient uncharacteristic skin rash and moderate lymph node enlargement may occasionally be observed. Signs of central nervous system (CNS) involvement may be pronounced and early, but more commonly they develop later during disease progression. Neurological symptoms consist mostly of seizures, hypotonia or hypertonia, ataxia, hemiplegia, or nonspecific signs of increased intracranial pressure. Nonspecific findings such as jaundice, edema, and failure to thrive as well as purpura and bleeding resulting from thrombocytopenia may be associated (Table 54.1).

PATHOLOGICAL FEATURES

The major histological finding is infiltration of the various organs by a nonmalignant activated lymphocyte population, mainly of the $CD8^+$ T-cell phenotype, associated with macrophage cell infiltration (Billiau et al., 2005; Farquhar & Claireaux, 1952; Henter et al., 1991a). The infiltrating lymphocytes and macrophages are most prominent in the interstitial and perivascular spaces of the organs. Activated macrophages may engulf erythrocytes, leukocytes, and platelets, their precursors, and cellular fragments, a process known as hemophagocytosis (Color Plate 54.IA). These cells appear "stuffed" with other blood cells. All organs may be infiltrated, predominantly the spleen, liver, bone marrow, lymph nodes, and CNS (Haddad et al., 1997; Henter & Nennesmo, 1997). Importantly, pathological analysis should be repeated if initially negative in the presence of strong clinical and biological suspicion of HLH.

When examined at an early stage, the white pulp of the spleen is often reduced in size and depleted of lymphocytes, whereas the red pulp is expanded as a result of the mononuclear cell infiltration. In the liver, portal tracts are the place of moderate to extensive lymphocytic infiltration; in the lymph nodes, sinuses are frequently involved and dilated (Henter

Table 54.1 CLINICAL AND BIOLOGICAL FEATURES OF HLH SYNDROME

CLINICAL MANIFESTATION	BIOLOGICAL MANIFESTATIONS
High fever	Cytopenia
Edema	Hypofibrinogenemia
Hepatomegaly	Hypertriglyceridemia
Splenomegaly	Elevated liver enzymes
Icterus, skin rash	Cerebrospinal fluid pleocytosis
Neurological symptoms	Low natural killer cell activity
	Presence of activated T lymphocytes and macrophages infiltrating various organs
	Hemophagocytosis by activated macrophages

Table 54.2 T LYMPHOCYTE AND MACROPHAGE ACTIVATION MARKERS DURING HLH SYNDROME

LYMPHOCYTE ACTIVATION	MACROPHAGE ACTIVATION
Expression of HLA DR$^+$, CD25$^+$, Fas$^+$	High serum level of TNF-α, IL-6, neopterin
High serum level of soluble CD8 and CD25	Hemophagocytosis
High serum level of IFN-γ	

et al., 1998). Hemophagocytic cells are predominantly found in the T-cell areas, frequently depleted of lymphocytes in the later stages of the disease. CNS infiltration begins generally in the meninges, then perivascular changes occur, leading to diffuse infiltration of the tissue and multifocal necrosis at a later stage.

LABORATORY FINDINGS

Viral infection, principally of the herpes group such as EBV, can be identified at the onset of the disease. However, in many cases viral, bacteriological, and serological investigations fail to implicate a known infectious agent.

At the onset of the disease, most patients present with anemia, thrombocytopenia, and, to a lesser degree, neutropenia. Leukopenia at first presentation is less frequent, and an initial leukocytosis is reported in 15 percent of patients. Nearly all patients become severely pancytopenic during disease progression. In the cerebrospinal fluid, moderate pleocytosis ($5–50 \times 10^6$/L), consisting mostly of lymphocytes and occasionally monocytes as well as an increased protein level, is observed in about half of the patients early in the course of the disease (Arico et al., 1996; Haddad et al., 1997; Janka, 1983). However, spinal fluid may be normal even in children with encephalitis. Hyperdense areas, atrophy, and brain edema may be found by magnetic resonance imaging or computed tomography scan, particularly later during the prolonged course of the disease.

Signs of liver dysfunction or cytolysis are constant findings and include hypertriglyceridemia, hyperbilirubinemia, elevated serum transaminases, elevated ferritin, hyponatremia, and hypoproteinemia. Coagulation abnormalities are common during active disease, particularly hypofibrinogenemia.

IMMUNOLOGICAL FINDINGS

The proliferative response of peripheral blood lymphocytes to phytohemagglutinin (PHA) and antigen is normal. However, markedly decreased natural killer (NK) cell activity is a consistent finding of inherited HLH associated in most of the conditions (see below) with a decrease in T-cell cytotoxic activity, a function that does not normalize during the remission phase. Activated CD8$^+$ T cells and, to a lesser extent, CD4$^+$ T cells are observed during the course of an active HLH. T-cell count may be transiently increased but then decreased, as with the other hematopoietic lineages. High levels of soluble CD8 and CD25 molecules as well as hypercytokinemia with elevated serum levels of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin (IL)-6, are striking features of HLH and reflect the T-lymphocyte and macrophage activation (Billiau et al., 2005; Henter et al., 1991b) (Table 54.2).

UNDERSTANDING THE PATHOPHYSIOLOGY OF HLH

Our understanding of inherited disorders leading to HLH has been revolutionized in the past 10 years by the molecular characterization of most of these disorders. This has shown that genes associated with inherited forms are part of the cytotoxic granule-mediated cell death pathway (see below) and shed light on a previously unsuspected role for this pathway in lymphocyte homeostasis (de Saint Basile & Fischer, 2001; Menasche et al., 2005). Importantly, they clearly show that T cells are the trigger for HLH, and gaining better control of T-cell activation is the best way to manage and control the disease.

CYTOTOXIC GRANULE-MEDIATED CELL DEATH PATHWAY

The granule-dependent cytotoxic pathway is a rapid powerful and iteractive mechanism adapted to the killing of virus-infected cells as well as tumor cells (reviewed in de Saint Basile & Fischer, 2001; Fischer et al., 2007; Menager et al., 2007; Voskoboinik et al., 2006). Cytotoxic T cells are activated by specific antigen recognition, whereas the cytotoxic activity of NK cells is initiated by specific activating receptors or combinations thereof and is inhibited by self MHC class I recognition. Cytotoxic T lymphocytes (CTLs) and NK cells contain cytoplasmic granules that can undergo regulated secretion of their content in response to external stimuli. These granules contain perforin (the central protein for CTL-mediated killing), granzyme, and other granule components (hence the

term "cytotoxic granules"). The process of cytotoxic granule secretion itself, which is triggered by target cell recognition, is characterized by a series of sequential events in the cytotoxic cells. First, the microtubule-organizing center (also called the "centrosome") is polarized toward the cell–cell contact, where an immunological synapse (IS) forms. Next, cytotoxic granules move toward the microtubule-organizing center, are released from the microtubules, and dock with the plasma membrane at the secretory domain of the IS. Cytotoxic granules then coalesce with endosomal exocytic vesicles. The coalesced granules then are primed and fuse with the plasma membrane, and the contents of the granules are released into the synaptic secretory cleft. Then, perforin and granzyme cooperate to mediate apoptosis of the target cell within a few minutes of receptor engagement. Not all granules are exocytosed, and the remainder are ready for a new target interaction and killing.

ROLE OF CYTOTOXIC ACTIVITY DEFICIENCY IN HLH PATHOGENESIS

The most obvious explanation for this is the persistence of antigen-presenting cells, which are not properly eliminated when cytotoxicity is impaired. Although T-cell–mediated recognition leads to T-cell activation and clonal expansion, lymphocytes deficient in cytotoxic function fail to kill the infected cells and thus to remove the source of antigen stimulation. Persistently activated T cells, which produce high quantities of cytokines (e.g., INF-γ), induce macrophage activation (Fig. 54.1). The sustained macrophage activation results in tissue infiltration and the production of high levels of IL-6, IL-18, and TNF-α, which play a major role in the various clinical symptoms and in tissue damage (Binder et al., 1998; Kagi et al., 1999; Matloubian et al., 1999; Nansen et al., 1999). In fact, there is a striking resemblance between the biological changes induced by inflammatory cytokines and the clinical and laboratory findings that characterize HLH (Henter et al., 1991b). However, the fact that, in most cases, HLH is not associated with a high load of the triggering infectious agent suggests that additional mechanisms may also play a role.

An alternative—not mutually exclusive—mechanism involves cytolysis having a direct function in killing T cells engaged in a given immune response. CTLs could either commit suicide or kill each other. The former mechanism is not compatible with a positive transdominant effect of functional CTLs observed following hematopoietic stem cell transplantation in patients with HLH (Ouachee-Chardin et al., 2006). It has indeed been observed that the presence of 20 percent of donor cells is sufficient to control HLH over at least 10 to 20 years after hematopoietic stem cell transplantation (Ouachee-Chardin et al., 2006). For a CTL to kill another CTL implies that it can recognize the proper Ag-MHC complex at its surface. This mechanism could be operating because, in vitro, it has been shown by several groups that, upon target-cell killing, CTLs can reap off parts of the target membranes that can be incorporated into their own plasma membrane (Huang et al., 1999; Stinchcombe et al., 2001b), making this cell a target for a sister CTL. The key question that is difficult to address experimentally is whether this mechanism takes place in vivo in a quantitative way that is sufficient to transform billions of CTLs into target cells within a few days. Regulatory T cells armed with the cytotoxic equipment were also reported (Grossman et al., 2004), which may participate in the contraction phase of a T-cell–mediated immune response. Ultimately, a regulatory role of cytotoxic NKT cells should also be considered based on the observation that human CD4- CD8aa NKT cells can kill in vitro antigen-presenting cells or T cells in a CD1d-restricted manner and thus contain T-cell expansion (Ho et al., 2004).

INHERITED CONDITIONS WITH OCCURRENCE OF HLH

FHL

FHL, inherited as an autosomal recessive disease, was first described by Farquhar and Clairaux as familial erythrophagocytic lymphohistiocytosis (Farquhar & Claireaux, 1952). Since then, the incidence of FHL has been estimated to be 1:50,000 births (Dufourcq-Lagelouse et al., 1999a; Henter et al., 1991c). Overwhelming HLH is the distinguishing and isolated feature in this disorder with no other associated signs, unlike the other inherited conditions. Symptoms of HLH are usually evident within the first 3 months of age and can even develop in utero or at birth (Lipton et al., 2004). In contrast, familial forms have been reported with

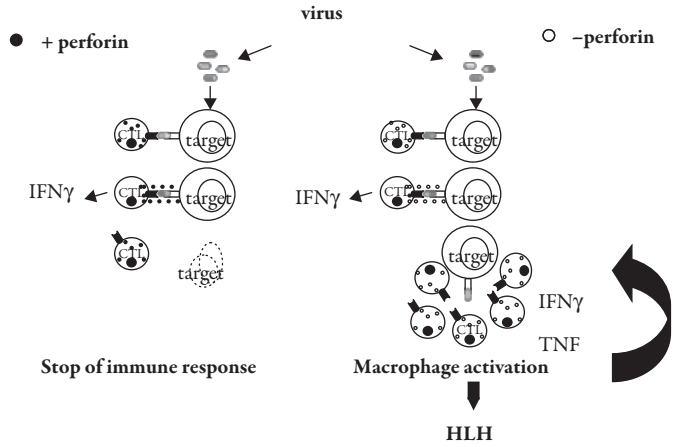

Figure 54.1 *Pathophysiology of HLH.* In response to a viral infection, antigen-specific cytotoxic T cells (CTLs) will expand, secrete soluble mediators (IFN-γ) that enhance immunity and interfere with viral replication, and mediate lysis of infected cells. These various mechanisms participate in downregulation of the immune response. In cytotoxic-deficient cells (perforin-deficient cells (– perforin) on the scheme), uncontrolled increased expansion of antigen-specific effectors occurs. Activated lymphocytes secrete high levels of INF-γ and induce a feedback loop on macrophages and T cells, which continuously activate each other and expand. High levels of inflammatory cytokines are secreted, including IFN-γ, TNF-α, and interleukins 6 and 18. Activated macrophages phagocytose bystander hematopoietic cells (hemophagocytosis). Activated lymphocytes and macrophages infiltrate various organs, resulting in massive tissue necrosis and organ failure.

a later onset (Arico et al., 1996; Henter et al., 1998; Janka, 1983). Several studies suggest that within a family, a tendency toward a similar age at onset is observed. HLH most often occurs in previously healthy young children, suggesting the need for an exogenous trigger prior to the onset of clinical manifestations. In susceptible children, infection with intracellular pathogens (viral and fungal, among others) is the most likely trigger for disease manifestation (Feldmann et al., 2002). HLH in FHL is invariably lethal unless treatment with allogeneic stem cell transplantation is performed (Jabado et al., 1997).

Molecular Basis of FHL

Previously, linkage analysis using homozygosity mapping in four inbred FHL families of Pakistani descent identified a locus (FHL1) on chromosome 9q21.3–22 (Ohadi et al., 1999). However, no causative gene has been so far associated with this locus. Association of this locus with FHL seems restricted to Pakistani families, although not all FHL cases in Pakistani families segregate with this locus (Feldmann et al., 2003). Using genome-wide linkage analysis, three additional loci have been identified on chromosomes 10q21–22 (FHL2) (Dufourcq-Lagelouse et al., 1999a), 17q25 (FHL3) (Feldmann et al., 2003), 6q24 (FHL4) (zur Stadt et al., 2005), and 19p13 (FHL5) and there is further evidence of additional genetic heterogeneity and of yet undefined gene(s) (unpublished data) (Table 54.3).

Perforin Deficiency in FHL2

The cytolytic effector perforin (PRF1), present in cytotoxic granules, is the first gene product identified as causing FHL (Goransdotter Ericson et al., 2001; Stepp et al., 1999) (Table 54.3). The perforin gene comprises three exons, of which only exons 2 and 3 are translated, encoding for the 555–amino acid polypeptide (Lichtenheld et al., 1988). As a consequence of perforin gene mutations, perforin protein expression is diminished to barely detectable in cytotoxic granules (Feldmann et al., 2002; Kogawa et al., 2002; Stepp et al., 1999), leading to defective cytotoxic activity. In normal cells, following release from lytic granules, perforin is thought to oligomerize in order to form pore-like structures in the target cell membrane, analogous to the C9 component of complement (Lukoyanova & Saibil, 2008; Stepp et al., 2000). Failure of perforin activity is etiologically linked to the development of FHL, and its deficiency accounts for one third of FHL cases (Stepp et al., 1999; Ueda et al., 2003; Zur Stadt et al., 2006). Over 50 different mutations in the perforin gene have been found in FHL2 patients; they consist of microdeletion, nonsense, or missense mutations. These mutations are distributed all along the sequence. Some perforin mutations recur in the same ethnic populations, which suggests common ancestors. For example, the Trp374 stop (1122G>A) appears to occur at high frequencies in Turkish families, the L364 frameshift (1090delCT) in the Japanese population, and the L17 frameshift (50delT) alteration in the African population (Lee et al., 2006). Some peculiar mutations of the perforin gene have been observed that specifically affect proteolytic cleavage and thus maturation of the protein (Katano et al., 2004), or its calcium-binding ability (Feldmann et al., 2005; Voskoboinik et al., 2004). Most of these mutations result in undetectable expression of perforin in lytic granules. Mutations only partially impairing perforin expression and function are rare but were shown to result in atypical (late-onset) disease. Among them the substitution Ala91Val, found with high frequency in healthy individuals (4 to 8 percent), was first considered as a neutral polymorphism. Further studies demonstrated that the A91V variant results in a partial loss (50 percent) of PRF1-dependent cytotoxicity and strongly suggested that the A91V polymorphism can predispose to atypical disease expression if inherited as a homozygote and can cause HLH if inherited with a second perforin allele with "null" activity (Voskoboinik et al., 2007). Recent reports also bring convincing evidence that some temperature-sensitive mutations in perforin may be associated with delayed FHL onset and predisposition to hematological malignancy (Chia et al., 2009).

Table 54.3 **AUTOSOMAL RECESSIVE DISORDERS ASSOCIATED WITH OCCURRENCE OF HLH**

	FLH_1	FLH_2	FLH_3	FLH_4	FLH_5	GS_2	CHS	HPS II
gene	unknown	PRF1	UNC13D	STX11	STXBP2	RAB27a	CHS1/LYST	AP3B1
locus	(9q21.3-22)	(10q21-22)	(17q25)	(6q24)	(19p13)	(15q21)	(1q42-43)	(5q14-1)
Protein/Function	?	perforin/ pore forming protein	Munc13-4/ priming factor	syntaxin 11/ membrane fusion	Munc18-2/ syntaxin binding protein	Rab27a/ tethening	Lyst/lysosomal fission-protein sorting	Ap3b1/ sorting of lysosomal protein
murin model	?	*prf1 -/-*	*Jinx*	*stx11-/ -*	not viable	*ashen*	*beige*	*pearl*
HLH	+	+	+	+	+	+	+	+ /- *
cytotoxic activity	?	-	-	+/-	-	-	-	-
hypopigmentation	-	-	-	-	-	+	+	+
specific keys features							giant granules	neutropenia

* only one case have developped HLH, who also carry an heterozygous Rab27a mutation

Munc13–4 Deficiency in FHL3

Patients whose disease is associated with FHL3 locus present with typical features of FHL and are indistinguishable from patients with perforin defect (FHL2). However, in patients with FHL3, perforin is normally expressed and functional. FHL3 was found associated with mutations in the gene *UNC13D* encoding for hMunc13–4, a member of the Munc13-UNC13 family (Feldmann et al., 2003) (Table 54.3) This gene contains 32 exons that encode a 123 kDa protein. Two calcium-binding (C2) domains separated by long sequences containing two Munc13-homology domains (MHD1 and MHD2) structurally characterize hMunc13–4. Most of the mutations so far identified in *UNC13D* are missense mutations, deletions, splice-site mutations, or nonsense mutations predicted to result in major changes in the protein (Marcenaro et al., 2006; Santoro et al., 2006; Zur Stadt et al., 2006; Sieni et al., 2011). Study of exocytosis of cytotoxic granules in lymphocytes from patients with FHL3 mutations showed that Munc13–4 was required for the release of the lytic granule contents of both CTLs and NK cells, but not for other secretory pathways, including the secretion of IFN-γ from TCR-activated lymphocytes (Feldmann et al., 2003; Marcenaro et al., 2006; Ueda et al., 2006). Thus, hMunc13–4 is an essential effector of the cytolytic granule pathway. Munc13–4–deficient lymphocytes can make normal contacts with target cells, stable conjugates, and polarize the lytic machinery as efficiently as do control lymphocytes. However, when Munc13–4 is lost in CTLs, cytotoxic granules dock at the membrane in the immunological synapse but are not released. This supports a role for Munc13–4 at a late step of this pathway in exocytosis, subsequent to docking. Munc13–4 is most probably required at a priming step of lytic granule secretion, following granule docking and preceding plasma granule membrane fusion (Feldmann et al., 2003; Stinchcombe et al., 2001b). An additional role of Munc13–4 upstream of its role as a priming factor at the IS was recently show. Munc13–4 is in fact required in the formation of a pool of endosomal vesicles that coalesce with cytotoxic granules before their exocytosis (Menager et al., 2007). Of interest, Munc13–4 is expressed in multiple cell types, including platelets and lungs; however, the phenotype of FHL3 patients is not different from that of patients with perforin deficiency. FHL3 accounts for about one third of the FHL cases.

Syntaxin 11 Deficiency in FHL4

Patients with FHL4 carry mutations in the syntaxin 11 gene (*STX11*) (zur Stadt et al., 2005), a member of the soluble N-ethylmaleimide sensitive factor attachment protein receptor present on target membrane (tSNARE) family of proteins involved in membrane fusion events (Table 54.3). All of the mutations in *STX11* so far reported are null mutations and most of them were identified in patients from Turkish origin, where they account for approximately 20 percent of the FHL patients (Zur Stadt et al., 2006). Although a defective cytotoxic activity of FHL4 patients' NK cells is clearly detected, defective cytotoxic activity of syntaxin 11-deficient CTLs are more difficult to detect by standard techniques. In addition, IL-2 stimulation partially restores cytotoxic NK-cell defects (Bryceson et al., 2007). Syntaxin 11 is thus another effector of the cytotoxic machinery required for the release of cytotoxic granules contents, likely by regulating membrane fusion events (Arneson et al., 2007). The precise step of the cytotoxic pathway regulated by syntaxin 11 remains to be characterized. The fact that this genetic form of FHL (FHL4) with partial NK-cell cytotoxic defect and those with severe impairment of this activity in both NK and T lymphocytes (FHL2 and FHL3) have indistinguishable phenotypes strongly suggests that the in vitro assays used to date do not, in fact, fully reflect the in vivo behavior of cytotoxic cells. Alternatively, additional mechanism(s) with impairment of NK-cell cytotoxic activity may operate in FHL4 (Zhang et al., 2008).

Munc18–2 Deficiency in FHL5

The most recently identified cause of FHL, FHL5, is a deficiency of the syntaxin-binding-protein-2 (*STXBP2)* gene, which encodes Munc18–2 (Cote et al., 2009; zur Stadt et al., 2009). STXBP2/Munc18–2 belongs to the SM family of fusion accessory proteins. These proteins are partners of SNARE protein, playing a complementary role in membrane fusion (Toonen et al., 2003; Südhof and Rothman, 2009). Like syntaxin 11, STXBP2/Munc18–2 is widely expressed. The various mutations identified in this gene affect protein stability (Cote et al., 2009; zur Stadt et al., 2009; Cetica et al., 2010). These mutations seems to be correlated with phenotype, in terms of age at onset and disease severity (Cote et al., 2009; Pagel et al., 2012). In addition to the classic FHL findings, atypical features including sensorineural hearing deficit, abnormal bleeding and severe diarrhea were reported (Pagel et al., 2012). Syntaxin 11 levels are very low in STXBP2/Munc18–2-deficient lymphoblasts and these two proteins can be coimmunoprecipitated. Thus, syntaxin 11 is probably the main partner of Munc18–2 in lymphocytes, requiring Munc18–2 for stable expression. Consistent with the pathophysiological features of FHL, Munc18–2-deficient NK cells have impaired cytotoxic activity that is partially restored by IL2 stimulation, as previously reported for syntaxin 11-deficient NK cells (Cote et al., 2009; zur Stadt et al., 2009; Cetica et al., 2010). A role for Munc18–2 in late stages of the exocytosis pathway is supported by the observation that the perforin-containing granules of Munc18–2-deficient NK cells are normally polarized towards cognate target cells, despite the impairment of exocytosis preventing them from releasing their contents (Cote et al., 2009). Thus, the same defective cytotoxic phenotype characterizes both syntaxin 11 and STXBP2 deficiencies, providing support for the existence of a functional interaction between these two proteins in the degranulation process.

GRISCELLI SYNDROME

GS (MIM 214450) is an autosomal recessive heterogeneous disorder characterized by pigmentary dilution, with a silvery-gray sheen of the hair and a typical pattern of uneven distribution of large pigment granules easily detectable

by light-microscopic examination (Color Plate 54.IIB) (Griscelli et al., 1978; Klein et al., 1994). Sun-exposed areas of patients' skin are often hyperpigmented, and microscopic dermis–epidermis junction analysis detects accumulation of mature melanosomes in melanocytes, contrasting with the hypopigmented surrounding keratinocytes (Griscelli et al., 1978). This typical pigmentary dilution is either associated with the occurrence of an HLH (GS2) (Ménasché et al., 2000) or primary neurological feature (GS1) (Pastural et al., 1997) or can be an isolated finding in rare patients (GS3) (Menasche et al., 2003b), defining three genetic forms of the syndrome (GS1, GS2, and GS3).

Clinical and Pathological Manifestations

The single most consistent cutaneous expression of partial albinism in patients with GS is a silvery-gray sheen to their hair (Klein et al., 1994; Menasche et al., 2003b). This is more obvious in patients with black hair but is also visible in patients with blond hair. Patients generally have lighter hair than that of their unaffected family members. Hypopigmented spots on the retinas have been described in some patients (Klein et al., 1994), but this is not a constant feature. In association with this characteristic pigmentary dilution, most patients develop a recurrent hemophagocytic syndrome (or accelerated phases) with features identical to those mentioned above for HLH (Table 54.1). Similarly, the onset of HLH syndrome in this condition is frequently triggered by an infection (Klein et al., 1994) and is characterized by hyperactivation and proliferation of T cells and macrophages, in association with fever, edema, hepatosplenomegaly, pancytopenia, coagulation abnormality, liver dysfunction, and features of hemophagocytosis. These patients have been defined as having GS type 2, which results from mutation in the Rab27a gene (*RAB27A*) (Table 54.3) (Ménasché et al., 2000).

Neurological manifestations are frequently observed in association with HLH in GS2 patients, as a result of brain infiltration by activated lymphocytes and macrophages (Schmid et al., 2009; Trottestam et al., 2009). However, in a few GS patients, severe and static neurological symptoms are noticeable since birth, without any sign of HLH, and consist of hypotonia, absence of coordinated voluntary movements, and severe psychomotor developmental delay (Sanal et al., 2002). These symptoms are similar to the neurological signs described by Elejalde et al. (1979). In this condition, CNS disorder is static and never improves with time. Patients presenting with the typical hypopigmentation of GS in association with this isolated severe neurological manifestation have been shown to carry mutations in the myosin VA gene (*MYO5A*), which defines GS1 (Pastural et al., 1997).

In addition, isolated hypopigmentation with typical hair and skin features of GS was recently observed in a subject, independent of *MYO5A* or *RAB27A* mutation. Hypopigmentation in this case resulted from mutation in the melanophilin gene (*MLPH*) (Menasche et al., 2003b), defining a third form of GS (GS3).

Molecular Basis

Genetic linkage analysis performed in several GS1 and GS2 families has enabled localization of both disease loci on the same 15q21 chromosome region. Mutations in either the *MYO5A* gene or *RAB27A* gene, two genes separated by less than 1.6 cM in this genetic region, account for the GS1 and GS2 phenotypes, respectively (Ménasché et al., 2000; Pastural et al., 2000). In addition, the melanophilin gene, located on chromosome 2q37.3, leads to GS3 when mutated. In this condition, the GS phenotype is restricted to the characteristic hypopigmentation of this condition (Menasche et al., 2003b).

Functional Aspects

Myosin VA is an unconventional myosin heavy chain implicated in vesicle transport in cells and is particularly abundant in neurons and melanocytes. It has the expected structure for a member of this family—that is, a globular head domain containing the ATP- and actin-binding sites; a "neck" domain, which is the site of calmodulin (or light-chain) binding; and a tail domain, which is thought to represent the cargo-binding domain (Fig. 54.2). Myosin VA acts as a dimer and moves cargo along actin filaments in a plus-end-directed manner, allowing their capture and accumulation at the periphery of the cells. Myosin VA is required for melanosome transport in melanocytes (Wu et al., 2002). Study in *dilute* mice has shown that a defect in myosin Va leads to concentration of melanosomes in the center of the melanocytes; they are also concentrated in dendrites and dendritic tips in wild-type mouse melanocytes. This defect also impairs processing of presynaptic vesicles in the peripheral regions of neurons and the distribution of smooth endoplasmic reticulum in Purkinje cell neurons (Takagishi et al., 1996).

Rab27a is expressed in melanocytes, peripheral leukocytes, platelets, and many other cells and tissue types, except in the brain (Chen et al., 1997). Each member of the Rab protein family has a characteristic intracellular distribution pattern, suggesting their unique function in transport. Like myosin VA, Rab27a co-localizes in part with melanosomes in melanocytes and its defect leads to the abnormal melanosome distribution observed in GS (Bahadoran et al., 2001). In addition, Rab27a is necessary for cytotoxic granule exocytosis and thus cytotoxic activity of T and NK cells, which has been shown to be defective in this group of GS2 patients (Ménasché et al., 2000). In cytotoxic cells, Rab27a can associate with a member of the synaptotagmin-like protein (SLP) family, SLP2a, which allows the docking of cytotoxic granules to the plasma membrane (Fig. 54.2) (Menasche et al., 2008).

Melanophilin, another effector of Rab27a, is specifically expressed in melanocytes (Fukuda, 2003). MLPH deficiency leads to the same defective transport of melanosomes as that resulting from *MYO5A* and *RAB27A* defects. Pigmentary dilution is indistinguishable among these three molecular defects. A specific role of the three proteins Rab27a, Myosin VA, and Mlph in the capture and actin-based transport of melanosomes to the periphery of the dendrites has been

Griscelli syndrome(GS)	Hypopigmentation	Primary neurological impairment	Hemophagocytic syndrome	Molecular defect
GS 1	+	+	–	MYO5A
GS 2	+	–	+	RAB27A
GS 3	+	–	–	Melanophilin (MLPH)

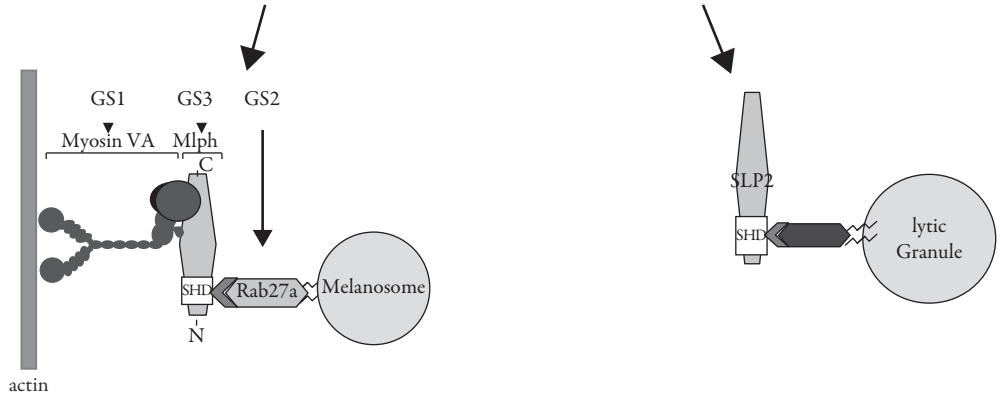

Figure 54.2 *Molecular defect leading to the three forms of Giscelli syndrome (GS).* A defect in Myosin VA, Rab27a, or Mlph leads to identical pigmentary dilution found in the three forms of GS. The formation of the heterotrimeric protein complex allows the transport of melanosome on actin fibers. Phenotypic expression of each genetic defect implies that organelle transport in melanocytes, neurons, and CTLs uses different effectors associated with Rab27a and Myosin VA. Slp2a is an effector of Rab27a in cytotoxic cells.

demonstrated. Analyses in mouse mutant models determined that Rab27a first associates with the membrane of melanosomes and then interacts with melanophilin, which recruits myosin Va through its tail region (Wu et al., 2002). Binding of the head domain of myosin Va to actin filaments links melanosomes with the peripheral actin network (Fig. 54.2).

Because of the role of myosin VA in brain tissue, however, only patients with myosin VA defect develop a primary severe neurological impairment with no immunological expression. In contrast, defective Rab27a expression is always associated with abnormal lymphocyte cytotoxic activity, which results in a lymphoproliferative syndrome, as observed in the case of the FHL caused by genetically determined perforin or hMunc13–4 deficiency (as discussed above). When neurological signs are observed in GS2 patients, they are the consequence of an accelerated phase and appear secondary to perivascular lymphohistiocytic organ infiltration.

Laboratory Findings

The light-microscopic examination and electron-microscopic findings of patients' hair and skin are characteristic. Hair shafts contain a typical pattern of uneven accumulation of large pigment granules instead of the homogeneous distribution of small pigment granules in normal hair (Color Plate 54.IIB). Fontana-stained silver sections of the skin show hyperpigmented melanocytes, contrasting with poorly pigmented adjacent keratinocytes, instead of the homogeneous distribution of melanin granules observed in melanocytes and surrounding keratinocytes in normal epidermis. Electron microscopy shows that the cytoplasm of melanocytes is filled with numerous mature stage IV melanosomes, predominantly around the nucleus, but have normal dendritic processes (Griscelli et al., 1978; Klein et al., 1994; Takagishi et al., 1996). These findings are consistently observed and identical in the three groups of patients (with *MYO5A*, *RAB27A*, or *MLPH* mutations).

Immunological abnormalities are restricted to patients with the Rab27a defect. Biological features of the accelerated phase are not specific to GS; they are identical to those observed in FHL patients. The capacity of lymphocytes and NK cells of these patients to lyse target cells is impaired or absent. This decrease in T- and NK-cell cytotoxicity, which results from an inability to secrete cytotoxic granules when *RAB27A* is not functional, is a constant feature in this group of patients (Ménasché et al., 2000). Patients have normal numbers of T, B, and NK cells as well as normal B and neutrophil functions, although decreased chemiluminescence and chemotactic responses have been reported (Klein et al., 1994). In addition, impaired skin reactions to tuberculin, streptokinase-streptodornase antigen, and *Candida* have been described in some cases. The lymphocytes proliferate in vitro in response to PHA, purified protein derivative (PPD), or *Candida*, and in mixed lymphocyte reaction (MLR). No immunological abnormalities have been observed in GS patients with a myosin VA or melanophilin defect. Myosin VA and melanophilin defects do not affect cytotoxic granule secretion, and these patients never develop an accelerated phase (Menasche et al., 2000, 2003b).

Mutation Analysis

RAB27A consists of seven exons, the first two being untranslated. It encodes a 221–amino acid polypeptide with a molecular mass of 25 kDa (Chen et al., 1997). Mutations in *RAB27A* have been characterized in about 100 independent patients (Ménasché et al., 2000; Zur Stadt et al., 2006; unpublished results). Very few missense mutations have been reported and

functionally analyzed (Menasche et al., 2003a). The other mutations are nonsense mutations, deletions, or splice-site alterations, all predicting an early protein truncation. In each case, the location of the stop codon predicts truncation of the consensus carboxylterminal motif of the protein, involved in Rab protein geranyl-geranylation, and thus should leave Rab27a protein in an inactive state.

The *MYO5A* gene contains an open reading frame of 1,855 codons that encodes a protein of 215 kDa (Mercer et al., 1991). Myosin VA is a member of the molecular motor molecules with structurally conserved heads followed by a neck domain, a tail region, and a globular C-terminal domain (Cheney et al., 1993). Alternatively spliced transcripts can be generated within the tail region of myosin VA. Most of the mutations identified in *MYO5A* lead to a predicted truncated protein (unpublished result). Alternative splicing within the tail region of myosin Va produces different spliced isoforms with tissue-restricted expression: in melanocytes, the majority of myosin VA transcripts contain the longest isoform of myosin VA, whereas brain transcripts contain a shorter isoform lacking exon F (Huang et al., 1998; Lambert et al., 1998; Pastural et al., 2000). In one patient, homozygous intragenic deletion of the MYO5A F-exon led to a phenotype restricted to typical hypopigmentation of GS without neurological symptoms, demonstrating that the F-exon of MYO5A is dispensable for neurological function (Menasche et al., 2003b).

The *MLPH* gene comprises seven exons spanning 2403 bp and encodes for a protein of 66 kDa. A homozygous R35W substitution was identified in the synaptotagmin-like protein homology domain (SHD) of the protein in one patient, which completely blocked Mlph interaction with the active form of Rab27a (Menasche et al., 2003b).

Genotype–Phenotype Correlation

As discussed above, there is a strict correlation between the type of gene affected (*RAB27A*/*MYO5A*/*MLPH*) and the phenotype displayed by patients with GS. Although pigmentary dilution is identical in these three groups of patients, only the patients with *RAB27A* mutations have decreased cytotoxic activity, resulting in the development of a hemophagocytic syndrome, whereas primary, severe, and irreversible neurological impairment characterizes patients with *MYO5A* mutations (Menasche et al., 2000, 2003b).

CHEDIAK-HIGASHI SYNDROME

Clinical and Pathological Manifestations

CHS (MIM 214500) is a frequently fatal, autosomal recessive genetic disorder characterized clinically by hypopigmentation of the skin and the hair, mild bleeding tendency, recurrent infections, neurological abnormalities, and early death from a so-called "accelerated phase" of the disorder that corresponds to the occurrence of an episode of HLH (Beguez-Cesar, 1943; Chediak, 1952; Higashi, 1954). A pathognomonic feature of the disease is the presence of intracellular giant organelles, which are seen in peripheral blood leukocytes and many other cell types (Color Plate 54.IB) (Spritz, 1999). In some patients, the diagnosis is considered only after the observation of these giant granules as an incidental finding on a peripheral blood smear. Most patients are diagnosed during the first decade of life, but in few patients with mild expression, the first diagnosis is made only in adulthood.

Dermatological manifestations in CHS show hair color varying from blond to dark but always exhibiting a silvery-gray tint, which is particularly noticeable in strong light (Stegmaier & Schneider, 1965). Microscopic examination of hair reveals the presence of clusters of melanin in the hair shaft (Color Plate 54.IIC) (Blume & Wolff, 1972; Windhorst et al., 1968). An increased red reflex, photophobia, and nystagmus in bright light can also be observed, which represent expressions of the ocular pigmentary dilution (Bregeat et al., 1966; Johnson et al., 1966). Patients' skin is less pigmented than their parents and siblings, and they are susceptible to severe sunburns. Some of these patients have chronic periodontal disease (Delcourt-Debruyne et al., 2000; Shibutani et al., 2000) or dermatological infections.

About 85 percent of patients develop HLH during the first decade (Page et al., 1962; Rubin et al., 1985; Spritz, 1985), which is frequently triggered by active EBV infection (Okano & Gross, 2000; Rubin et al., 1985). Neurological manifestation can be part of the episode of HLH, ranging from seizures to coma (Table 54.1). Nevertheless, 10 to 15 percent of patients have a relatively mild early clinical course and may survive to adulthood with few or even no severe infections and no sign of the accelerated phase. Patients who survive the childhood period, with or without complications of the hemophagocytic syndrome, often develop neurological manifestations, including mental deficiency or progressive intellectual decline, peripheral neuropathy with tremor, muscle weakness, stiffness, clumsiness, abnormal gait, and foot drop (Misra et al., 1991; Uyama et al., 1994). There is now strong evidence that these neurological manifestations, which are the first recognized manifestation of the disease in some adult patients, are primary rather than resulting from brain infiltration by activated lymphocytes and macrophages during previously undiagnosed HLH episodes (Tardieu et al., 2005). Many of these patients with the clinically milder adolescent or adult forms of CHS appear to have mutations in the *LYST* gene that allow some residual function of the CHS1 polypeptide (Karim et al., 2002).

Laboratory Findings

Giant intracytoplasmic granulations are the hallmark of this disorder and occur in all granulated cells, including hematopoietic cells (Color Plate 54.IB) and melanocytes as well as fibroblasts, renal tubular, neurons, and Schwann cells (Blume & Wolff, 1972; White, 1966; Windhorst et al., 1968). They display endosomal/lysosomal characteristics and appear preferentially distributed to the perinuclear region of the cells (Burkhardt et al., 1993; Jones et al., 1992). This pigmentary disturbance results from the abnormal aggregation of melanin into giant melanosomes and their inability to transfer melanin to the adjacent keratinocytes (Fukai et al., 1993; Griscelli et al., 1978; Windhorst et al., 1968).

Both T and NK cells present a defective cytotoxic activity, which results from their inability to secrete cytolytic proteins

stored in their giant granules (Katz et al., 1982; Klein et al., 1980). Biological features, which characterized the occurrence of the so-called accelerated phase of the disease, are indistinguishable from those during an episode of HLH (see above) (Tables 54.1 and 54.2).

Molecular and Functional Aspects

The human *LYST* (also known as *CHS1*) gene is enormous, consisting of 55 exons spanning more than 222 kb in chromosome 1q43 (Karim et al., 2002) The *CHS1/LYST cDNA* (13.5 kb) (Nagle et al., 1996) encodes for a huge cytosolic protein of 425 kDa (3801 amino acids (aa)) (Barbosa et al., 1996; Perou et al., 1997). CHS1/LYST belongs to a family of proteins, called the *BEACH* family, that share the three same C-terminal domains: a Pleckstrin-Homology domain (PH) (Jogl et al., 2002), a BEACH domain (named after BEige And Chediak-Higashi) (Nagle et al., 1996), and WD40 repeats. However, the exact function of these domains remains unknown, though they are supposed to play a role in the binding of protein partners (Gebauer et al., 2004; Jogl et al., 2002). Most of the functional information on CHS1/LYST comes from the studies of other members of the BEACH family, which define them as vesicle trafficking regulatory proteins (Martens & McMahon, 2008). The CHS1/LYST protein also contains a series of ARM (ARMadillo) (Peifer et al., 1994) and HEAT repeat motifs thought to mediate membrane associations and vesicle transport (Andrade & Bork, 1995). Recently, a lectin-like domain has been identified in the N′ terminal region of the CHS1/LYST and of other BEACH proteins (Burgess et al., 2009). It could be involved in oligosaccharide binding associated with protein traffic and sorting along the secretory pathway, especially in relation with components of the vesicle fusion machinery.

Spectrum of Mutations

Considering the length of the *LYST* gene, mutation analysis in CHS patients is a difficult task. Interestingly, most of the mutations so far reported lead to truncated proteins, either by nonsense mutation or frameshift mutations (Barbosa et al., 1996; Certain et al., 2000; Karim et al., 2002; Nagle et al., 1996). Similar homozygous mutations have been found associated with typical or, less commonly, with milder clinical courses, even within the same family (Certain et al., 2000). Thus it seems likely that factors other than *LYST* gene mutations may influence the clinical expression of CHS. Nevertheless, one important genotype–phenotype correlation has emerged. Only four patients have been described with *LYST* missense mutations resulting in amino acid substitutions (Karim et al., 2002). Two of these, with homozygous missense substitutions, had mild, adult-onset CHS. Overall, it appears that most but not all patients with null-mutant *LYST* alleles manifest a clinically severe course, whereas many or all patients with missense mutant alleles manifest a relatively mild clinical course. Maternal uniparental isodisomy for the entire chromosome 1 was also reported in a case of CHS (Dufourcq-Lagelouse et al., 1999b).

HERMANSKY-PUDLAK SYNDROME

Clinical and Pathological Manifestations

The term Hermansky-Pudlak sydrome (HPS) (MIM203300) is used to define a group of at least eight human autosomal recessive genetic disorders (HPS-1 through -8) characterized by oculocutaneous albinism and storage pool deficiency due to defects in the formation of melanosomes and platelet dense granules, respectively (Gunay-Aygun et al., 2004; Hermansky & Pudlak, 1959; Nazarian et al., 2008; Wei, 2006). The storage pool deficiency leads to easy bruising, hemorrhagic diathesis as a result of prolonged bleeding times, and lysosomal ceroid storage (Dell'Angelica et al., 2000; Huizing et al., 2000; Oh et al., 1996). Patients have a tyrosinase-positive albinism, and microscopic analysis of the hair shaft does not allow the detection of clusters of pigment, contrasting with the pigmentation features characterizing hair from CHS and GS patients. Like other forms of albinism, HPS patients have reduced visual acuity and nystagmus.

HPS type 2 (HPS2, MIM 203300) is the only form of HPS that is also associated with immunodeficiency, mainly increased susceptibility to infections due to congenital neutropenia and defective cytotoxic activity of T and NK lymphocytes (Clark et al., 2003; Enders et al., 2006; Jung et al., 2006). Some of these patients may also display lung fibrosis, facial dysmorphism, developmental delay, or hepatosplenomegaly (Huizing & Gahl, 2002; Shotelersuk et al., 2000).

Molecular and Functional Aspects

HPS2 results from mutation in *AP3B1*, the β chain of the adaptor protein-3 (AP-3) complex (Table 54.3) (Dell'Angelica et al., 1999). Only few missense and nonsense mutations or deletions in *AP3B1* have been so far reported (Enders et al., 2006; Huizing & Gahl, 2002; Shotelersuk et al., 2000). AP3 is a ubiquitous cytoplasmic complex, consisting of four different subunits, that shuttles cargo proteins from the trans-Golgi and a tubular-endosomal compartment to endosome-lysosome-related organelles (Dell'Angelica et al., 1997a, 1997b). AP3 thus functions in protein sorting to lysosomes. Defects in the β3A subunit disrupt the complex and all subunits are rapidly degraded. Misrouting of lysosomal proteins such as CD107 and CD63 to the cell membrane has been found in fibroblasts, CTL clones and platelets (Clark et al., 2003; Kurmi et al., 2012). Similarly, CD1b, which is sorted by AP3, but not other CD1 isoforms, fails to gain access to lysosomes. Protein missorting may explain several features of the HPS2 phenotype. Aberrant subcellular targeting of neutrophil elastase likely contributes to the observed neutropenia (Benson et al., 2003; Massullo et al., 2005), while missorting of tyrosinase contributes to the pigmentation disorder (Honing et al., 1998). Presentation of lipid antigens from bacteria is reduced due to less efficient sorting of CD1b to the lysosome, which may also participate in the increased susceptibility to infections of HPS2 patients (Sugita et al., 2002).

Spectrum of Mutations

Some 12 HPS2 patients with unique *AP3B1* mutations have been reported, including missense, nonsense and deletion mutations (Chiang et al., 2010; Dell Angelica et al., 199; Enders et al., 2006; Fontana et al., 2006; Huizing et al., 2002; Jung et al., 2006; Kurnik et al., 2012; Wenham et al., 2010). One of these patients had a homozygous nonsense mutation in exon 8 of *AP3B1* (p.R302X) and in addition was heterozygous for a *RAB27A* mutation; he died at age 3 years of fulminant HLH (Enders et al., 2006). Only one other patient with a *RAB27A* mutation developed symptoms of HLH (Wenham et al., 2010).

Impaired Cytotoxic Activity and HLH in HPS2

In the absence of AP3, cytotoxic T- and NK-cell–mediated killing was shown to be significantly impaired, with the presence of enlarged cytotoxic granules unable to move along microtubules toward the MTOC when the cytotoxic cell recognizes a target (Clark et al., 2003; Enders et al., 2006; Jung et al., 2006). Although all HPS2 patients tested have defective cytotoxic activity, only two cases were so far reported with the development of HLH (Enders et al., 2006; Wenham et al., 2010). It is unclear whether the additional heterozygous *RAB27A* mutation observed in one of the two patients contributed to this complication (Enders et al., 2006). However, pearl mice, representing a murin model of HPS2, when infected with lymphotic choriomeningitis virus, developed transiently key features of HLH but fully recovered even if an additional Rab27a mutation was knocked-in (Jessen et al., 2013)

Thus, a key clinical question that remains regarding HPS2 is whether the cytotoxicity defect in HPS2 predisposes to HLH and therefore represents an indication for preemptive hematopoietic stem cell transplantation. Long-term follow-up will be necessary to try to answer this question.

STRATEGIES FOR DIAGNOSIS OF INHERITED HLH

Distinguishing primary from secondary forms of HLH is an important task, not only for genetic counseling but also for determining appropriate therapeutic interventions. Onset of HLH at a young age should instigate the search for a genetic cause. Microscopic analysis of the hair shaft is an easy and reliable test to diagnose GS and CHS. In both conditions, pigmentation dilution is characteristic, with larger clumping of pigment in the hair shafts of GS than CHS patients (Color Plate 54.II). Carriers of these syndromes have normal pigmentation. The presence of giant intracytoplasmic granules in all cells from the hematopoietic lineage is a hallmark of CHS and is easy to identify in a blood smear (Color Plate 54.IB), ensuring a rapid diagnosis. If pigmentation dilution orients toward GS, sequencing of the *RAB27A* gene allows confirmation of the diagnosis of GS2. In the absence of HLH, molecular diagnosis of GS is important to rule out potential Rab27a deficiencies, which should be treated by allogeneic stem cell transplant. In CHS, given the length of the *LYST* gene, mutation screening is not used as a routine test for diagnosis and genetic counseling. Unambiguous diagnosis of this condition can be performed based on the characteristic hypopigmentation of the hair shaft and the presence of intracellular giant granules, without the need for further genetic testing. However, for genetic counseling of families, segregation analysis of polymorphic markers linked to the *CHS* locus on chromosome 1q43.2 in the family can be used (Barrat et al., 1996). In non-consanguineous families, this approach requires availability of DNA samples from both parents and the patient to determine the affected haplotype in the family. When the parents are related, the identification of a shared haplotype at the *CHS* locus in the parents may overcome the lack of a DNA sample from the patient. When HLH is not associated with hypopigmentation, the biggest difficulty lies in differentiating between the primary (inherited) disease (FHL) and a secondary HLH disease. A positive family history, with previously affected family members and/or consanguinity, is highly suggestive of an inherited form. The availability of biological samples from family members (parents, siblings) greatly helps the molecular diagnosis of genetic causes by rapid determination of the polymorphic markers segregating with the disease locus. However, the lack of a family history is not a reliable criterion to exclude FHL. Study of the cytotoxic activity of T and/or NK lymphocytes (Bryceson et al., 2007) is a consistent test to diagnose the genetic forms of HLH. NK- and T-cell cytotoxicity is defective in most of the FHL cases resulting from either a perforin defect a Munc13–4 defect, Syntaxin 11 defect or Munc18–2 defect. Perforin defects can be rapidly identified by the analysis of perforin expression in resting cytotoxic cells using an immunofluorescence study. In fact, the great majority of the mutations so far identified in FHL2 dramatically affect perforin detection. Sequencing of the perforin gene will confirm the diagnosis of FHL2. In the other group, sequencing of the *UNC13D* gene, Syntxin 11gene or STXBP2 gene allows identification of FHL3. FHL4 or FHL5 respectively. Some patients with STXBP2 mutation also display early and severe enteropathy requiring parenteral nutrition (Pagel et al., 2012), likely resulting from a prime role of Munc18–2 in gut epithelial cells. In the rest, the genetic cause is not yet characterized. Defective lymphocyte cytotoxic activity is the signature of a primary genetic cause of HLH. Although not discussed in this chapter, the diagnosis of XLP should be excluded in male patient developing HLH during EBV infection, by sequencing of the *SH2D1A* and *XIAP* genes, the two molecular causes of XLP so far characterized (Nichols et al., 1998; Rigaud et al., 2006) (See Chapter 44).

TREATMENT AND PROGNOSIS

Therapy of the inherited forms of HLH consists of two phases (Henter et al., 2007; Mahlaoui et al., 2007; Schmid et al., 2009): the aim of the first phase is to reach remission; in the second phase, hematopoietic stem cell transplantation will be

envisaged. Basically, two treatment protocols are used today to strive for remission:

- Chemotherapy (etoposide, VP-16) in combination with corticosteroids and cyclosporin A (according to the HLH1994 and HLH2004 studies) (Henter et al., 2007)
- Immunotherapy (antithymoglobulin, ATG) in combination with corticosteroids and cyclosporin A (according to the recommendation of the European Society of Immunodeficiencies [ESID]) (Jabado et al., 1997; Mahlaoui et al., 2007; Schmid et al., 2009)

These treatments induce remission in approximately 80 percent of patients. However, they also induce a severe immunosuppression that may lead to infectious complications. Etoposide is furthermore known to occasionally induce immediate hepatic and mucosal toxicity, increasing the risk of therapy-related secondary neoplasia. Infusion of ATG, on the other hand, can be associated with hemodynamic instability at the beginning of the treatment.

Intrathecal application of methotrexate, combined with hydrocortisone or high-dose steroids, should be considered in patients with severe neurological involvement.

ANIMAL MODELS

The natural mouse mutant *pearl* represents the murine counterpart of human HPS2 (Feng et al., 1999). Pearl mice show hypopigmentation, abnormal lysosomal secretion, and abnormal platelet dense granules, in association with mutations in the *Ap3b1* gene subunit of the AP3 adaptor complex. Similar findings are seen in an engineered *Ap3b1* knockout mouse strain (Yang et al., 2000). Following LCMV infection, pearl mice transiently develop the features of HLH associated with moderate defect in CTL cytotoxicity (Jessen et al., 2013). Interestingly, neutrophil counts appear normal in *Ap3b1*-deficient mice, whereas dogs with mutation in *AP3B1* have cyclic neutropenia (Benson et al., 2003).

CHS has been described in many mammalian species (Windhorst & Padgett, 1973), including mink, cattle (Padgett et al., 1964), cats (Kramer et al., 1977), foxes, killer whales, rats (Nishimura et al., 1989; Ozaki et al., 1994), and mice (Lutzner et al., 1967). Several natural mouse mutants of lyst exist (*Lyst*-mutated) (*souris* and *beige*), which have been characterized in molecular terms (Barbosa et al., 1996; Nagle et al., 1996). The phenotype of the souris and *beige* mouse corresponds to human CHS in virtually all aspects, including pigmentary dilution, reduced chemotactic and bactericidal activity of granulocytes (Gallin et al., 1974), reduced NK and T-lymphocyte cytotoxic function (Saxena et al., 1982), enlarged melanosomes in pigment cells, and enlarged lysosomes in many cell types (Novak et al., 1984; Windhorst & Padgett, 1973). Only the *souris* but not the *beige* strain develop features of HLH following LCMV infection, which may be explain by the difference in degree of cytotoxicity defect between these two mutants (Jessen et al., 2011).

The three forms of GS naturally occurred in three different mutants—*dilute* (d), *ashen* (ash), and *leaden* (lead) mice, which result from Myosin Va, Rab27a, and Mlph deficiency, respectively. All murine mutants present with identical pigmentary dilution and normal pigment granule synthesis but abnormal melanosome transport. The melanosomes are localized mainly in the perinuclear region of the melanocytes (Provance et al., 1996; Wilson et al., 2000). *Dilute* null mice also show a neurological defect, characterized by opisthotonus and ataxia, which results in the death of the animal 2 to 3 weeks after birth (Dekker-Ohno et al., 1996; Takagishi et al., 1996). Isolated pigmentation dilution characterizes the *leaden* mice and results from a deletion of seven residues in the SHD of the melanophilin gene (Matesic et al., 2001). *Ashen* mice exhibit the same pigmentary dilution, but in association with defective cytotoxic activity (Haddad et al., 2001; Stinchcombe et al., 2001a). As in GS2, this defective function results from the role of Rab27a in cytotoxic granule exocytosis. A splicing defect resulting from a single transversion in the exon 4 donor splice site, activation of a cryptic downstream site, and abnormal Rab27a transcript was identified in these mice (Wilson et al., 2000).

Two FHL animal models have been generated. The perforin-deficient mouse (*Prf1*$^{-/-}$) (Kagi et al., 1994) was obtained by disruption of the perforin gene and is the mouse counterpart of FHL2. The Munc13–4 deficient mouse, also known as *Jinx* mouse, was obtained by ENU-induced germline mutation that introduced a splice defect in the *Unc13d* sequence, resulting in an early truncation of the Munc13–4 protein in this mouse model of FHL3 (Crozat et al., 2007).

The mouse models of human HLH that have identified defective cytotoxic activity (i.e., the *Prf1* knockout mice, the *Jinx* mice, the *ashen* mice, the syntaxin-11 mice (Sepulveda et al., 2012; Kögl et al., 2012), the pearl mice and the *souris* mice) are all healthy when maintained under standard housing conditions, although they do have defective granule-dependent cytotoxic activity. When challenged with certain strains of viruses, such as LCMV, every one of these knock-out mice developed an overreactive, virus-specific T-cell response, although with different severity and duration, with splenomegaly, cytopenia (anemia, neutropenia, and thrombocytopenia), and increased levels of IFN-γ, TNF-α, and other inflammatory cytokines (Crozat et al., 2007; Jordan et al., 2004; Pachlopnik Schmid et al., 2008). Activated macrophages with hemophagocytosis can be found in the liver of these mice. Thus in this setting, mouse models of FHL2, FHL3, FHL4, ,GS2, CHS and HPS2 develop a pathology identical to that of human HLH. Most of the *Prf1*$^{-/-}$ mice die within a few weeks as a consequence of HLH, whereas the others murin models show a substantially better survival rate. Several studies have shown that death of these infected deficient mice results not from a defect in viral clearance but from persistence of the bulk of activated CD8^{+} cells and the deleterious action of the cytokines they secrete (Badovinac et al., 2003; Jordan et al., 2004; Kagi et al., 1999; Matloubian et al., 1999; Rossi et al., 1998). A critical role of the CD8^{+} T lymphocyte population and the high level of IFN-γ this population produces have been shown to be critical for the occurrence of HLH in these mouse models, since depletion of the CD8 population or neutralization of INF-γ prevents the immune disease. The therapeutic administration of an antibody that blocks IFN-γ was shown to induce recovery from HLH in two genetic models tested, the *Prf1* knockout and the *ashen*

(Rab27a knockout) mice, as based on prevention of death of the former and correction of the various features of HLH in both murine models (Pachlopnik Schmid et al., 2009).

CONCLUDING REMARKS AND FUTURE CHALLENGES

The past few years have seen great progress in understanding the molecular bases of the various inherited forms of HLH (FHL, GS, CHS). As a result, genetic identification of the defect has generally translated into improved diagnosis and genetic counseling of these conditions. Likewise, discovery of the causal genes for these diseases has resulted in a better understanding of the pathophysiology of HLH. Analyses of the mouse models of HLH strongly suggested a primary role of the lymphocyte activation and IFN-γ production in the excessive macrophage activation and the proinflammatory cytokine production, which lead to disease pathogenesis. Similarly, there has been important progress in the treatment of these inherited conditions, largely brought about by the improved management of patients undergoing hematopoietic stem cell transplantation, and improvement of immunosuppressive treatment during the remission phase. Nevertheless, considerable challenges remain to find an efficient but less toxic therapy, as suggested by IFN-γ neutralization experiment in murine models. In addition, a few inherited FHLs remain to be molecularly identified.

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55.

GENETICALLY DETERMINED DEFICIENCIES OF COMPLEMENT COMPONENTS

Kathleen E. Sullivan and Jerry A. Winkelstein

OVERVIEW

The Nobel Prize in Physiology was given to the Belgian microbiologist Jules Bordet in 1919 for his description of the complement system. Bordet purified proteins from guinea pig serum and identified a heat-stable fraction with antibody activity and a heat-labile fraction that "complemented" the antibody fraction in a lytic assay.

In the past century, we have come to understand this "complementing function" well. The complement system is a group of 14 proteins, comprising the complement cascade, and more than 10 regulatory proteins. In addition, there are at least seven receptors that mediate the biological functions of the complement cleavage products. Complement proteins are very abundant in serum and rise with infection or inflammation. Nearly 5 percent of all serum proteins are complement proteins, and this number can rise to 7 percent of serum proteins in inflammatory states. The abundance reflects its important function, as does its conservation throughout evolution. Complement has been evolutionarily conserved, and the primary role of complement throughout evolution relates to "marking" of pathogens with C3b, a process called opsonization. The term "opsonization" is derived from the Greek word for condiment or delicacy and refers to the facilitation of phagocytosis by neutrophils. The neutrophils engulf bacteria more readily when they are coated with complement. This role of complement as an opsonin is its most important role in host defense.

The majority of serum complement is produced in the liver, although C1q, properdin, and C7 are produced predominantly by myeloid cells and factor D is produced by adipocytes and is also known as adipsin (Anthony et al., 1989; Schwaeble et al., 1994; Scoazec et al., 1994; Wilkison et al., 1990; Ziccardi, 1983). For this reason, liver disease is associated with a significant defect in serum complement levels. The tissue-specific and inducible expression of most complement proteins is regulated at the level of transcription.

This chapter will address the biochemistry of the complement cascade and discuss inherited disorders of complement components. The complement cascade's main role is to deposit C3b on the surface of microbes and to generate small-molecule mediators to regulate inflammation (Walport, 2001a, 2001b). Direct killing of bacteria, clearance of apoptotic cells, and the regulation of the adaptive immune system are also important roles.

The nomenclature of the complement cascade is unfamiliar to many people, and a few of the more common terms are defined in Table 55.1. The nomenclature follows certain patterns, with the classical pathway components generally indicated with an uppercase C followed by a number that roughly correlates with the position in the cascade (C4 appears out of order). Alternative pathway members are generally referred to as a "factor" and are designated with a letter (factor B, factor D, factor H). Lectin pathway members start with the letter M (for mannose). As protein fragments are cleaved off, they are given lowercase letter identifiers, with the "a" most often designating the smaller fragment (the exception is C2a, which is larger than C2b). In some cases, the two fragments can be further cleaved, and those smaller fragments are named with additional lowercase letters. When a cleavage product is inactive, it is preceded by the letter "i."

INTRODUCTION TO THE COMPLEMENT SYSTEM

A simple model for the organization of the complement cascade has three activation arms: the classical pathway,

the lectin activation pathway, and the alternative pathway (Fig. 55.1). These three pathways cleave the central protein, C3, and allow it to bind to the nearest surface, usually a pathogen. A complex including activated C3 can cleave C5, leading to the assembly of the terminal components. These proteins catalyze the formation of a pore, generated by C8 and stabilized by C9.

THE CLASSICAL PATHWAY

The classical pathway is activated primarily by immune complexes. A subtle conformational change occurs when antibody engages antigen, which renders the antibody molecule capable of interacting with C1 (Gal et al., 1994; Sim & Reid, 1991). Not all antibody is equivalent in terms of its ability to activate complement: only IgG and IgM activate complement, and IgM is much more efficient than IgG. In addition, not all isotypes of IgG are equivalent: IgG3 is the most efficient, followed by IgG1 and IgG2. IgG4 is not able to activate complement. In addition to immune complexes, C-reactive protein, serum amyloid P, β-amyloid, DNA/chromatin, cytoskeletal filaments, SIGN-R1, and some pathogens can activate the classical pathway (Riley-Vargas et al., 2005; Sjoberg et al., 2006).

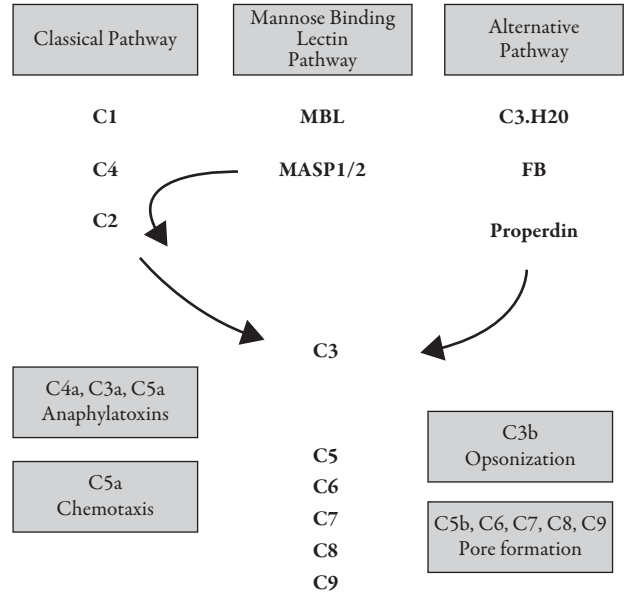

Figure 55.1 The complement system consists of three activation arms, converging on the cleavage of C3 and the activation of the terminal components. The classical pathway is activated predominantly by immune complexes, while the lectin activation arm and the alternative pathway are truly innate and are activated directly by pathogens. The fluid-phase cleavage products shown in the gray boxes on the left have anaphylatoxic and chemotactic activities. The membrane-bound components listed in the gray boxes on the right are more directly involved in pathogen killing.

Table 55.1 DEFINITIONS

Classical pathway	**C1, C4, C2, C3, and the terminal components**
Alternative pathway	**Factor B, Factor D, properdin, and the terminal components**
Lectin activation pathway	**MBL, MASP1, MASP2, C3, and the terminal components**
Anaphylatoxins	**C3a, C4a, C5a. These are mediators of smooth muscle contraction, degranulation of mast cells, enhanced neutrophil aggregation, increased vascular permeability.**
Opsonization	**Renders a particle more easily phagocytosed**
C3 tick-over	**This term is used to describe spontaneous C3 hydrolysis.**
Membrane attack complex (terminal components):	**C5, C6, C7, C8, C9**
CH50	**The assay is used to define the dilution of serum capable of lysing 50% of the sensitized sheep red cells. This assay measures the intactness of the classical pathway through the terminal components.**
AH50	**The assay is used to define the dilution of serum capable of lysing 50% of nonsensitized rabbit red cells. This assay measures the intactness of the alternative pathway through the terminal components.**

The C1 complex is made up of C1q (which in turn is made up of six polypeptide chains) and two C1r and two C1s proteins. Interaction of C1 with immune complexes leads to a conformational change in C1 that allows autoactivation of C1r. C1r cleaves the two molecules of C1s, which cleaves C4 into C4a and C4b (not to be confused with the genetically distinct C4A and C4B genes). C4b is a highly reactive molecule that binds to the pathogen surface near the antibody–C1 complex (Law et al., 1984). The C4b–$C1s_2$ complex cleaves C2 into C2a and C2b. The smaller C2b is released and the C2a becomes incorporated into the growing complex. C3 binds to this complex and is cleaved by C2a, with C3a being released into the surrounding space and C3b binding to the pathogen surface.

The small fluid-phase cleavage products are biologically active. C4a, C3a, and C5a are termed anaphylatoxins (Schlaf et al., 2004; Weigle et al., 1982), and each binds a specific receptor, which leads to mast cell degranulation and neutrophil aggregation. Histamine from mast cells drives increased vascular permeability and vasodilatation (Hawlisch et al., 2005; Weigle et al., 1982). C5a is the most important of the anaphylatoxins, acting as both an anaphylatoxin and as a chemotactic factor for neutrophils (Robbins et al., 1987). Thus, within minutes of an antibody binding to a pathogen, the complement system has ensured that neutrophils are recruited to phagocytose and kill the pathogen and have enhanced the phagocytosis of the pathogen by coating it with C3b. The anaphylatoxins increase

blood flow to the site and increase vascular permeability such that additional complement proteins and antibodies can enter the tissue. C5a serves to direct neutrophils to the location of the pathogen. It is therefore no surprise that deficiencies of classical pathway components are associated with an increased predisposition to infection with bacteria.

THE ALTERNATIVE PATHWAY

The alternative pathway does not require the existence of preformed antibody, and it and the lectin activation pathway constitute the truly innate activation arms of the complement cascade (Pangburn, 1989a, 1989b). Alternative pathway activation relies on a biochemical quirk of C3. C3 spontaneously hydrolyzes in serum at a low rate (Pangburn & Muller-Eberhard, 1983). This spontaneous hydrolysis is often referred to as "C3 tick-over." The hydrolyzed C3 undergoes a conformational change that allows it to interact with factor B. Factor D cleaves factor B only when complexed with hydrolyzed C3. C3bBb is the alternative-pathway C3-converting enzyme, which is stabilized by properdin, and this enzymatic complex cleaves additional C3 into C3b and C3a (Nolan et al., 1991). Factor H on our own cells displaces factor B from C3b and catabolizes C3b, preventing activation of complement on our own cells (Fig. 55.2). Activation proceeds on surfaces such as mannose or N-acetyl glucosamine-rich pathogen surfaces, where factor H does not bind.

In summary, the alternative pathway is generally activated through the recognition of oligosaccharide and charge differences common to pathogens. The alternative pathway exploits the instability of the native C3 molecule and, on pathogen surfaces, nucleates a complex of C3bBb that cleaves additional C3.

THE LECTIN ACTIVATION PATHWAY

Mannose binding lectin (MBL) recognizes oligosaccharides specific to pathogens (Childs et al., 1989; Matsushita & Fujita, 1995; Sastry et al., 1989). Mammalian glycoproteins are generally decorated with galactose and sialic acid, not recognized by MBL, while MBL avidly binds to oligosaccharides associated with bacteria, yeast, and parasites such as mannose, N-acetylglucosamine, fucose, and glucose (Childs et al., 1989). Upon binding to the carbohydrate ligand, MBL activates two associated enzymes, MASP1 and MASP2. The arrangement of these enzymes with MBL is quite similar to the C1q-C1r-C1s arrangement. MASP2 cleaves C4 and MASP1 cleaves C3. Once C4b is bound to the pathogen surface, the remaining protein–protein interactions are identical to those in the classical pathway. MBL also binds to agalactosyl IgG with high affinity (Malhotra et al., 1995). This unusual IgG is produced primarily at sites of inflammation, and this antibody could activate both the classical pathway and the lectin activation pathway.

In summary, the lectin activation pathway represents an important arm of the innate immune system. This pathway is activated directly by pathogen oligosaccharides. The C3 convertase produced in the lectin activation pathway is identical to that of the classical pathway and the inflammatory mediators perform the same functions.

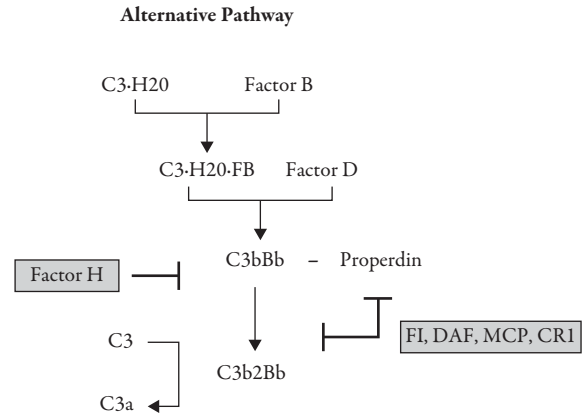

Figure 55.2 The alternative pathway is activated when factor B binds hydrolyzed C3. This complex either is cleaved by factor D, when a pathogen surface supports activation, or is inactivated by factor H on self-tissues. Properdin stabilizes C3bBb and in the presence of additional C3 can promote the formation of the C5 convertase complex, $C3b_2Bb$.

THE MEMBRANE ATTACK COMPLEX

Once C3 is cleaved by any of the activation arms described above, it becomes a part of the next enzymatic complex, the C5 convertase. This C5 convertase will be either C4b2a3b (classical and lectin activation pathways) or $C3b_2Bb$ (alternative pathway). The cleavage of C5 follows the typical pattern, with the larger fragment becoming attached to a surface and the smaller fragment diffusing into the fluid phase. C5b binds to C6 and C7, and they collectively serve to anchor the complex to the lipid bilayer (Halperin et al., 1993; Salama et al., 1983). C5b, which remains more external than the other components, binds directly to C8, which disrupts the physical integrity of the membrane and leakage of cytoplasmic proteins, and ions begin to occur. The addition of multiple molecules of C9 leads to the formation of true pore, as opposed to a leaky patch (Bhakdi & Tranum-Jensen, 1986). Nucleated cells can repair membrane damage and are coated with regulatory proteins, which prevent the lytic activity of the terminal components. Thus, it is often the red cells that are affected by complement activation clinically.

In summary, the terminal components, also known as the membrane attack complex, serve to induce lysis of a target. They are most efficient at lysing gram-negative bacteria.

RECEPTORS AND BIOLOGICAL FUNCTIONS

Receptors for complement mediate many of the biological functions of complement (Table 55.2). The G-protein–coupled anaphylatoxin receptors are widely expressed on mast cells, basophils, eosinophils, neutrophils, platelets, endothelial cells, and smooth muscle cells (Martin et al.,

Table 55.2 COMPLEMENT RECEPTORS

RECEPTOR	LIGAND	ROLE	COMMENTS
CR1	C3b, C4b, iC3b	Immune complex clearance, phagocytosis	Four allelic forms differ in size, CD35
CR2	C3d, C3dg, iC3b	B-cell activation	CD21
CR3	iC3b, C3d, C3b ICAM-1	Neutrophil adhesion, phagocytosis	β2 integrin, 165 kD α chain, 95 kD β chain, CD11b/CD18
CR4	iC3b, C3b	Neutrophil adhesion	β2 integrin, 150 kD α chain, 95 kD β chain, CD11c/CD18
CRIg	C3b, iC3b	Intravascular pathogen clearance	
C1qRp	C1q, MBL, surfactant protein A	Phagocytosis	126 kD, CD93
cC1qR	C1q, MBL, surfactant protein A	Phagocytosis	Recognizes collagen domain
C3aR	C3a >>> C4a	Increases vascular permeability, mast cell degranulation, chemotaxis	48 kD protein
C5aR	C5a, C5a desArg	Chemotaxis, mast cell degranulation, increases vascular permeability	43 kD protein, CD88

1997). These receptors mediate histamine release, aggregation of neutrophils, stimulation of mucus release from goblet cells, and activation of macrophages (Takahashi et al., 1996). In the face of an infection, these functions may all be viewed as protective (Fig. 55.3). The vasodilatation acts to deliver additional neutrophils to the site of inflammation, mucus release may facilitate clearance of organisms, activation of macrophages enhances phagocytosis, and aggregation of neutrophils reflects enhanced transendothelial migration into the affected tissue.

The C5a receptor can also mediate neutrophil chemotaxis. Thus, the early inflammatory response, mediated by small-molecule mediators, serves to deliver increased blood flow to the area and increases extravasation of antibody and complement into the tissues, and then C5a provides directionality for the recruited neutrophils (Fig. 55.3). This is a very efficient system to direct a response to a focal infection; however, in overwhelming sepsis, high levels of serum C5a can cause neutrophil aggregation, blockage of pulmonary vessels, and adult respiratory distress syndrome (ARDS). C5a also appears to be one of the major contributors to disseminated intravascular coagulation in sepsis (Czermak et al., 1999).

C1q receptors mediate phagocytosis and can enhance microbicidal activity. They also appear to be important for the clearance of apoptotic cells. The CR1 (CD35) receptor is widely expressed and primarily binds C3b, C4b, and iC3b (Medof et al., 1982; Weiss et al., 1989). Its main functions are the clearance of immune complexes to prevent unwanted inflammation and enhancement of phagocytosis. CR1 also has a regulatory role in the inactivation of C3 and the destabilization of the C3 and C5 convertase complexes (Wilson et al., 1987). The CR2 receptor (CD21) is the receptor for the Epstein-Barr virus and is expressed on B cells, follicular dendritic cells, epithelial cells, and some T cells (Cooper et al., 1990; Marquart et al., 1994).

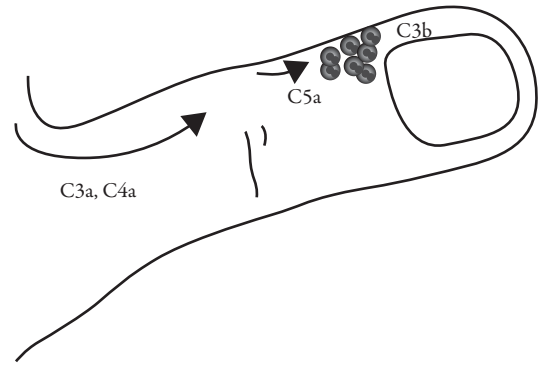

Figure 55.3 Complement plays multiple roles in ensuring the delivery of cells and proteins to sites of infections. The anaphylatoxins lead to vasodilatation and vascular leak, thus ensuring the delivery of cells and proteins to the site. C5a provides a chemotactic gradient for neutrophils and C3b acts as an opsonin to facilitate neutrophil phagocytosis.

Its main function is as a B-cell costimulatory molecule (Ahearn et al., 1996; Fischer et al., 1996; Marquart et al., 1994). Antigen linked to C3d activates B cells much more strongly than pure antigen (Dempsy et al., 1996).

CR3 and CR4 are both members of the β2 integrin family (Wagner et al., 2001). These receptors bind iC3b and C3b. The CR3 receptor also binds C3d. These receptors act primarily as adhesion molecules and recognize the opsonized pathogen. The CRIg receptor is the primary receptor mediating clearance of complement-tagged bloodborne pathogens (Wiesmann et al., 2006).

A typical bacterial infection would activate the complement cascade, and recognition of that act by specific cell-surface receptors would facilitate clearance of the pathogen. The anaphylatoxin receptors would enhance blood flow to the site and augment local concentrations of antibody and

Table 55.3 COMPLEMENT REGULATORY PROTEINS

PROTEIN	LOCALIZATION	FUNCTION	COMMENTS
C1 inhibitor	Serum	Binds to C1r and C1s and dissociates the C1 complex	105 kD
C4 binding protein	Serum	Cofactor for factor I cleavage of C4b	550 kD
Factor I	Serum	Cleaves C3b and C4b	
Factor H	Serum	Defines activator surface	
Decay accelerating factor (DAF)	Ubiquitous/cell membrane	Dissociates both C3 and C5 convertases	GPI-anchored CD55
Membrane cofactor protein (MCP)	Hematopoietic cells except erythrocytes	Cofactor for C3b cleavage by factor I	CD46
C8 binding protein	Most hematopoietic cells	Binds to C8 and prevents interaction with C9	GPI-anchored, also known as homologous restriction factor
CD59	Hematopoietic cells, endothelial cells, epithelial cells, glomerular cells	Inhibits the membrane attack complex	GPI-anchored, also known as HRF20

complement. C5aR would recruit neutrophils, CR1 and C1q would enhance phagocytosis, and the CR3 receptor would act as the major opsonic receptor. CR2 would act to improve antibody production to the pathogen. This beautifully integrated system plays a substantial role in host defense, and deficiencies of complement components demonstrate the important function of complement.

REGULATION OF COMPLEMENT ACTIVATION

The regulators of complement are divided into fluid-phase regulators and membrane-bound regulators (Table 55.3). C1 inhibitor is a fluid-phase inhibitor, and it regulates C1 by binding to C1s and C1r, leading to dissociation. It is thought that C1 inhibitor acts on MBL and MASP1/2 through a similar mechanism. C1 inhibitor has important roles inhibiting factor XII (Hageman factor) and prekallikrein of the contact system of coagulation (Cicardi et al., 2005; Davis, 2004), functions of paramount importance for the clinical features of C1 inhibitor deficiency.

C4 binding protein is another fluid-phase regulator of complement, and it acts to displace C2a and dissociate the classical pathway convertase (Gronski et al., 1988; Hessing et al., 1990). Factor I, alongside factor H, regulates the alternative pathway (Discipio & Hugli, 1982; Isenman, 1983; Kinoshita & Nussenzweig, 1984; Masaki et al., 1992). Factor H identifies nonactivator surfaces though the recognition of nonpathogen oligosaccharides and displaces Bb from C3b on those surfaces. Factor I inactivates C3b by cleaving it to iC3b, and its activity is enhanced in the presence of factor H. Together, these two regulators ensure that the spontaneous activation of the alternative pathway remains at a low level and prevents attachment of the complex to our own cells.

Factor I also acts to inhibit C4b from the classical pathway (Hardig et al., 1997; Masaki et al., 1992). In this setting, it interacts with C4 binding protein and cleaves C4b into C4c and C4d. C4 binding protein has an interesting structure and regulates the complement cascade as well as the coagulation cascade, as is true for C1 inhibitor.

The membrane-bound regulators of complement consist of the ubiquitous 70kD molecule termed DAF, membrane cofactor protein (MCP, CD46), C8 binding protein, and CD59 (Hansch et al., 1988; Lublin et al., 1986, 1987, 1988; Masaki et al., 1992; Rollins et al., 1991; Taguchi et al., 1990). DAF serves to dissociate the C3 convertase, and MCP serves as a cofactor for factor I cleavage of C3b and C4b. CD59 directly inhibits the membrane attack complex. Several receptors also act as complement regulatory proteins, and in these cases, the regulatory activity may be viewed as termination of that specific complement function after the signal has been delivered.

THE MECHANISMS UNDERLYING THE CLINICAL MANIFESTATIONS OF COMPLEMENT DEFICIENCIES

The clinical expression of genetically determined deficiencies of the complement system is variable and depends on the role of the deficient component in normal host defense and inflammation (Tables 55.4 and 55.5). Individuals with genetically determined deficiencies of complement components often have an increased susceptibility to infection or systemic lupus erythematosus (SLE). Less frequently, complement-deficient patients have a phenotype related to dysregulated vascular tissues.

INFECTION

Complement is especially important during the first few hours of infection (Winkelstein et al., 1975) and in containing the infection at its initial site (Bakker-Wondenberg et al., 1979).

Table 55.4 INHERITED COMPLEMENT DEFICIENCIES

DEFICIENCY	CHROMOSOMAL LOCATION	NUMBER OF CASES REPORTED	CLINICAL FEATURES, DIAGNOSTIC STRATEGY
C1q	1p36.12	10–100	SLE, infections, CH50 near zero
C1r/s	12p13	10–100	SLE, infections, CH50 near zero
C4	6p21.3	10–100	SLE, infections, CH50 near zero
C2	6p21.3	Many	SLE, infections, some asymptomatic, CH50 near zero
C3	19p13.3–13.2	10–100	Infections frequent and severe, glomerulonephritis, CH50/AH50 near zero
Factor D	19p13.3	<10	*Neisseria*, AH50 near zero
Factor B	6p21.3	<10	*Neisseria*, AH50 near zero
Properdin	Xp11.3–11.23	>100	*Neisseria*, AH50 near zero
MBL	10q11.2–21	Millions	Most asymptomatic, MBL assay required
C5	9q33	10–100	*Neisseria*, CH50/AH50 near zero
C6	5p13	>100	*Neisseria*, CH50/AH50 near zero
C7	5p13	>100	*Neisseria*, CH50/AH50 near zero
C8	1p32, 9q34.3	>100	*Neisseria*, CH50/AH50 near zero
C9	5p13	Many	*Neisseria*, CH50/AH50 diminished
Factor I	4q25	10–100	*Neisseria*, hemolytic uremic syndrome, C3 may be diminished, many require mutation analysis
Factor H	1q32	10–100	*Neisseria*, hemolytic uremic syndrome, C3 may be diminished, many require mutation analysis
MCP	1q32	<10	Hemolytic uremic syndrome, mutation analysis required
C1 inhibitor	11q12–13.1	Many	Angioedema, C1 antigen and functional levels
CR3/CR4	16p11.2	>100	Leukocyte adhesion deficiency, very severe systemic infections, lack of pus, flow cytometry
CD59	11p13	<10	Paroxysmal nocturnal hemoglobinuria, flow cytometry
DAF	1q32	10–100	Inab phenotype, blood typing
C4bp	1q32	<10	Autoimmunity, mutation testing

If dissemination should occur, complement contributes to the clearance of microbes from the bloodstream (Helmy et al., 2006; Hosea et al., 1980). Furthermore, complement plays an important role in resistance to infection in both the "immune" host with high levels of antibody and in the "nonimmune" host with little if any antibody.

Although studies in experimental animals have shown that the complement system has the potential to contribute to the host's defense against a wide variety of bacteria, fungi, and viruses, clinical experience would suggest that the greatest susceptibility of complement-deficient patients is to bacterial infections (Figueroa & Densen, 1991; Ross & Densen, 1984). The kinds of bacteria that most commonly cause infections in complement-deficient patients reflect the specific role of the missing component in normal host defense. For example, the major cleavage products of C3 (C3b and C3bi) are important opsonins. Therefore, patients with a deficiency of C3 have a markedly increased susceptibility to infection with those bacteria for which opsonization is the primary mechanism of host defense (e.g., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Haemophilus influenzae*). Patients with a deficiency of C1, C4, or C2 also have an increased susceptibility to these same encapsulated bacteria, since these components are necessary for the activation of C3 via the classical pathway. However, they do not appear to be as susceptible as patients with C3 deficiency, since their alternative pathway is intact and able to activate C3.

Although bloodborne, systemic infections such as bacteremia and meningitis are the most common bacterial infections reported in complement-deficient patients (Figueroa & Densen, 1991; Ross & Densen, 1984), localized infections such as sinusitis, otitis, and pneumonia have also been seen. Infections in complement-deficient individuals are usually caused by the common encapsulated bacteria to which normal hosts are also susceptible, such as the pneumococci, streptococci, and meningococci. However, systemic infections with unencapsulated strains of pathogenic bacteria such as the meningococci, which are extremely rare in normal individuals, have been described in a number of complement-deficient individuals (Hummel et al., 1987).

Activation of the terminal components, C5 to C9, results in the assembly of the membrane attack complex and the

Table 55.5 COMPLEMENT COMPONENT DEFICIENCIES: GENETIC BASIS

COMPONENT	GENE NAME	INHERITANCE PATTERN
C1q	*C1QA, C1QB, C1QG*	AR
C1r/C1s	*C1R/C1S*	AR
C4	*C4A, C4B*	AR
C2	*C2*	AR
C3	*C3*	AR
C5	*C5*	AR
C6	*C6*	AR
C7	*C7*	AR
C8A/B	*C8A/C8B*	AR
C8G	*C8G*	AR
C9	*C9*	AR
Factor B	*BF*	AR
Factor D	*DF*	AR
Factor H	*HFI*	AR and AD
Factor I	*IF*	AR and AD
MCP	*MCP*	AR and AD
Properdin	*PFC*	X-linked recessive
C4bp	*C4BPA/C4BPB*	Unknown
C1 inhibitor	*C1NH*	AD
DAF	*DAF*	AR
CD59	*CD59*	AR
MBL	*MBL*	AD and AR

AD, autosomal dominant; AR, autosomal recessive.

generation of bactericidal activity for gram-negative bacteria. Therefore, patients with deficiencies of one of the terminal components have an increased susceptibility to infection with those gram-negative bacteria, such as *Neisseria meningitidis*, that are most susceptible to the bactericidal activities of the complement system. As expected, infections with gram-positive bacteria, such as the pneumococci, are not seen with any greater frequency in patients with deficiencies of a terminal component because these bacteria are not susceptible to the bactericidal activity of the complement system.

Some clinical features of meningococcal infections are different in complement-deficient individuals from those of meningococcal infections in individuals with an intact complement system. It has been hypothesized that once the infection is contained with antibiotics, complement-mediated inflammation contributes to the severity and sequelae (Figueroa & Densen, 1991; Platonov et al., 1993; Ross & Densen, 1984).

SYSTEMIC LUPUS ERYTHEMATOSUS

Complement plays a role in tolerance, immune complex clearance, and clearance of apoptotic cells (Botto et al., 1998; Carroll, 2004; Navratil & Ahearn, 2000; Schifferli et al., 1986). These three mechanisms are believed to underlie the susceptibility of patients with C1, C4, and C2 deficiencies to SLE. As cells undergo apoptosis, intracellular constituents are reorganized and appear on the surface of the cell in blebs. Autoantigens targeted in patients with SLE, such as SSA and/or SSB, are often found on the surface in these blebs (Casciola-Rosen et al., 1994), rendering visible a normally invisible antigen.

There is a hierarchy of risk for SLE among the complement components. For example, less than half of patients with C2 deficiency and nearly 100 percent of patients with C1 deficiency develop SLE (Walport et al., 1997). In contrast, fewer than 2 percent of patients with deficiencies of C5, C6, C7, C8, or C9 have SLE. The hierarchy is partly explained by the ability of individual components to participate in immune complex clearance (Schifferli & Peters, 1983; Schifferli et al., 1985) and the clearance of apoptotic cells (Mevorach et al., 1998). C1 and C4 appear to be the most important for tolerance (Carroll, 2004), and this may explain why deficiencies of these two components are the most strongly associated with SLE.

A variety of other autoimmune diseases, including discoid lupus, dermatomyositis, scleroderma, anaphylactoid purpura, vasculitis, and membranoproliferative glomerulonephritis, have also been reported in patients with complement deficiencies (Figueroa & Densen, 1991; Ross & Densen, 1984). The mechanisms underlying these associations and the true frequencies are not known.

There are some clinical differences between the SLE seen in patients with C2 deficiency and the SLE seen in individuals without inherited complement deficiencies. The SLE seen in C2-deficient patients often has its onset in early adulthood, is frequently characterized by a prominent annular photosensitive dermatitis, and may have relatively mild pleuropericardial and renal involvement. In addition, it is not uncommon for C2-deficient patients to have low (or absent) titers of antibodies to nuclear antigen and/or native DNA (Glass et al., 1976; Meyer et al., 1985; Provost et al., 1983). In contrast, the prevalence of anti-Ro antibodies is reported to be higher than in non–C2-deficient patients with lupus (Jonsson et al., 2007; Provost et al., 1983). The SLE seen in patients with C1q deficiency tends to have severe end-organ effects and is usually associated with positive titers to nuclear antigen (Bowness et al., 1994; Vassallo et al., 2007).

HEMOLYTIC UREMIC SYNDROME

Complement regulatory proteins prevent the activation of complement on host tissues. The vasculature represents a particularly dangerous location, as all the cells are in constant contact with the complement proteins. Hemolytic uremic syndrome occurs due to defects in complement regulatory proteins and an inability to protect fenestrated endothelium in the glomerulus from complement-mediated damage (Pangburn, 2002). The anionic nature of the basement membrane can support complement activation. Microtrauma arises frequently due to the high oncotic pressure, and the

basement membrane is able to support complement activation if not protected. Complement-mediated destruction of the renal vasculature is the main mechanism of disease.

EPIDEMIOLOGY OF COMPLEMENT DEFICIENCIES

Infections and SLE are common clinical manifestations of genetically determined complement deficiencies. Accordingly, a number of studies have attempted to estimate the incidence of complement deficiencies in these disorders to determine how frequently complement deficiencies contribute to their pathogenesis and to evaluate the utility of screening certain groups of patients for complement deficiencies.

INFECTION

The prevalence of complement deficiencies is significantly higher in individuals with systemic meningococcal infections than in the general population. The frequency of complement deficiencies in sporadic cases of systemic meningococcal infections has been estimated to be between 1 and 18 percent (Ellison et al., 1983; Leggiadro & Winkelstein, 1987; Merino et al., 1983; Platonov et al., 1993; Rasmussen et al., 1987; Swart et al., 1993). In general, the prevalence is even greater in those patients who have had recurrent meningococcal disease (40 percent) (Nielsen et al., 1989b; Platonov et al., 1993), a positive family history of meningococcal disease (10 percent) (Nielsen et al., 1989b), or an unusual serotype of the meningococcus (20 to 50 percent) (Fijen et al., 1989). Chronic meningococcemia may also be associated with complement deficiencies. On the basis of these studies, it appears reasonable to screen any patient with a systemic meningococcal infection for a complement deficiency. Since there is a relatively high risk for recurrence, early identification of complement deficiency in patients with systemic meningococcal infections may prevent subsequent meningococcal infections.

The evidence that screening patients with systemic pneumococcal, streptococcal, or *H. influenzae* infections for complement deficiencies is less compelling (Densen et al., 1990; Ekdahl et al., 1995; Rasmussen et al., 1987; Rowe et al., 1989). Although sepsis and meningitis caused by these organisms are very common among patients with inherited complement deficiencies (Figueroa & Densen, 1991; Ross & Densen, 1984), one study demonstrated only a single complement-deficient patient in a series of 389 patients with bacteremia and/or meningitis caused by bacteria other than the meningococcus (Densen et al., 1990).

SYSTEMIC LUPUS ERYTHEMATOSUS

The prevalence of homozygous C2 deficiency is significantly higher in patients with SLE than in the general population. Genetically determined C2 deficiency is found in approximately 1 in 10,000 (0.01 percent) Caucasian individuals in the general population (Ruddy, 1986; Sullivan et al., 1994). In contrast, the prevalence of homozygous C2 deficiency in large series of patients with SLE has varied between 0.4 and 2.0 percent (Glass et al., 1976; Hartung et al., 1989; Sullivan et al., 1994). Although a number of patients with genetically determined C2 deficiency have also had other rheumatic diseases (Figueroa & Densen, 1991; Ross & Densen, 1984), there have been no studies that have documented a significantly increased prevalence of C2 deficiency in these disorders. Patients with early complement component deficiencies have a much higher risk of dying than typical SLE patients; therefore, screening for complement deficiency among all SLE patients may be warranted (Jonsson et al., 2005). Screening of SLE patients with an early onset of SLE or with a history of infections is more clearly indicated.

HEMOLYTIC UREMIC SYNDROME

Classical hemolytic uremic syndrome is due to *Shiga* toxins elaborated by bacteria. Pneumococcal infections can also cause a hemolytic uremic syndrome through a less-well-characterized mechanism. Atypical hemolytic uremic syndrome differs from classical hemolytic uremic syndrome because it is not related to clear-cut bacterial infections. Among patients with atypical hemolytic uremic syndrome, approximately 20 percent will have mutations in factor H. Mutations in MCP account for approximately 10 percent of cases, and another 10 percent of cases are ascribed to factor I mutations (Richards et al., 2007). Factor B and C3 mutations have been seen rarely. There are also factor H-related proteins that have been implicated in atypical hemolytic uremic syndrome, but the role of these proteins is not well understood. Two important aspects of these defects in regulatory proteins are that penetrance is incomplete and most mutations are heterozygous.

DISORDERS ASSOCIATED WITH COMPLEMENT DEFICIENCY

Most genetically determined complement deficiencies are inherited in an autosomal recessive fashion (Table 55.5). Properdin deficiency is X-linked and C1 inhibitor deficiency is autosomal dominant (heterozygous). Inherited complement component disorders are typically associated with a CH50 or AH50 of near zero and can be specifically defined in reference laboratories using a complementation system whereby patient sera are added to mixes generated with a single missing component. Mutation studies can be helpful for regulatory protein deficiencies but are not clinically warranted for intrinsic cascade proteins.

C1 DEFICIENCY

C1 is a complex of C1q, C1r, and C1s. C1q interacts with the antibody and has a tulip-shaped structure composed of three distinct proteins: C1QA, C1QB, and C1QC. C1r and C1s are embedded in the stem of the tulip and are the active enzymatic components of the complex. Patients with C1q deficiency (MIM 120550, 120570, 120575) present

nearly uniformly with early-onset SLE (Botto & Walport, 2002; Hannema et al., 1984; Slingsby et al., 1996). The autoantibody profile is similar in these patients compared to other SLE patients, although anti-dsDNA antibodies may be somewhat less common (Bowness et al., 1994; Walport et al., 1998). Clinically, the patients have more dramatic cutaneous and central nervous system (CNS) manifestations than in the typical patient with lupus, and the disease may be more severe. It is also believed that the lupus seen in C1q-deficient individuals is less steroid-responsive and has an earlier age of onset (Bowness et al., 1994; Walport et al., 1998). Patients with C1q deficiency also have an increased rate of infection, and this no doubt relates to compromised opsonization and a decrease in B-cell costimulation.

The molecular defects causing C1q deficiency are diverse, although there is a nonsense mutation at amino acid position 186 of C1qA that is common among C1q-deficient patients from Turkey (Berkel et al., 2000; Petry et al., 1996). C1q deficiency is not restricted to a specific ethnic heritage and has been identified in people of European, Asian, and Arabic heritage. Mutations in each of the three chains have been identified. In one case with a premature termination codon and undetectable A chain, B and C polypeptide chains were also absent, which suggests that C1A must be present for the formation and secretion of C1q (Petry et al., 1995). Because there are many mutations causing C1q deficiency, carrier detection and prenatal testing rely on the identification of each individual mutation in affected family members. Allele-specific polymerase chain reaction (PCR) has been used to identify carriers of the common Turkish mutation.

Fewer than 20 individuals with C1r deficiency (MIM 216950) have been described (Dragon-Durey et al., 2001; Inoue et al., 1998; Lee et al., 1978; Rich et al., 1979). Typically, C1r levels are markedly reduced (1 percent of normal) and C1s levels are 20 to 50 percent that of normal (Lee et al., 1978; Loos & Colomb, 1993). The mutations in patients with C1r deficiency have not been identified, but the fact that 7 of the first 12 patients identified with C1r deficiency have been of Puerto Rican descent suggests that there may be a founder effect.

C1s deficiency is less common than C1r deficiency. In these few cases, C1s has been markedly reduced, while C1r levels were 50 percent that of normal (Suzuki et al., 1992), a finding suggesting that neither monomer is stable in the absence of the other. Three mutations have been identified in patients with C1s deficiency (MIM 12058). One patient had a 4 bp deletion in exon 10, and two others had nonsense mutations in exon 12 (Dragon-Durey et al., 2001; Endo et al., 1999; Inoue et al., 1998).

C4 DEFICIENCY

There are two distinct genes for C4, termed C4A and C4B (MIM 120810, 120820). They are highly homologous, although C4A binds more avidly to protein while C4B binds more avidly to carbohydrate. Within each C4 gene, there can be deletions or duplications or simple inactivating mutations (Ballow et al., 1979; Fredrikson et al., 1991). One to two percent of the general population and up to 15 percent of patients with SLE have complete C4A deficiency. While C4A deficiency is a risk factor for SLE, the severity of the disease is often less in patients with C4A deficiency compared to complement-sufficient hosts (Colten, 2002; Howard et al., 1986; Meyer et al., 1985; Moulds et al., 1993; Welch et al., 1998). One to two percent of the population has complete C4B deficiency, but up to 15 percent of patients with invasive bacterial disease are C4B-deficient (Bishof et al., 1990). In contrast to the common partial deficiencies, complete C4 deficiency due to four inactive alleles is quite rare. Well over 50 percent of the C4-deficient individuals have SLE (Colten, 2002; Colten & Rosen, 1992; Mascart-Lemone et al., 1983). Cutaneous manifestations are common and severe, and the age of onset is often quite early. The renal disease has the typical appearance, although immunofluorescence for C4 is absent (Fig. 55.4). The usual array of autoantibodies are found, although anti-dsDNA antibodies may be somewhat less frequent in C4-deficient individuals compared to normal hosts. Infection appears to be a significant problem for patients with C4 deficiency, and infection is the major cause of death (Figueroa & Densen, 1991; Ross & Densen, 1984). The mechanisms underlying the predisposition to infection are probably related to impaired opsonization and a compromised B-cell response to antigen.

The mutations associated with complete C4 deficiency are diverse. Gene conversion accounts for the majority of C4B null alleles and a 2 bp insertion accounts for the majority of C4A null alleles; however, extensive mutation analyses have not been done in subjects from diverse racial backgrounds (Yang et al., 2004).

C2 DEFICIENCY

C2 deficiency (MIM 217000) is the most common of the inherited complement component deficiencies in Caucasians. Although rare in some ethnic groups, in Caucasians it is found with a frequency of 1:10,000. Most C2-deficient individuals are asymptomatic, in contrast to those patients with C4 and C1 deficiency. Between 20 and 40 percent of C2-deficient individuals will develop SLE (Figueroa & Densen, 1991; Jonsson et al., 2005; Ross & Densen, 1984). The age of onset is early adulthood, as is true for SLE in the general population. Although cerebritis, nephritis, and arthritis are less common in C2-deficient SLE patients compared to the typical SLE patient, cutaneous manifestations appear to be more common (Jonsson et al., 2007). Anti-Ro antibodies are extremely common in C2-deficient patients with SLE, although anti-dsDNA antibodies are infrequent (Vandersteen et al., 1982). Other autoimmune disorders have been described in patients with C2 deficiency although there may be an element of ascertainment bias, as CH50 assays are commonly run on patients with autoimmune disorders.

Over 95 percent of C2-deficient individuals are homozygous for a 28 bp deletion at the end of exon 6, which causes a splicing abnormality and premature termination of transcription (Johnson et al., 1992; Sullivan et al., 1994; Truedsson et al., 1993). The 28 bp deletion is associated with a conserved MHC haplotype: HLA-B18, BF*S, C2*Q0, C4A*4, C4B*2, DR2. The gene frequency of this deletion has been found to

be 0.005 to 0.007 percent in Caucasian populations (Sullivan et al., 1994; Truedsson et al., 1993), a frequency consistent with previous estimates of the prevalence of homozygous C2 deficiency of approximately 1:10,000 (Ruddy, 1986). In addition to the common 28 bp deletion, a 2 bp deletion has been found that results in a premature stop codon (Wang et al., 1998), and two different missense mutations have been identified that impair secretion of mature C2 (Wetsel et al., 1996). Detection of the 28 bp deletion through PCR has been used for both carrier detection and prenatal diagnosis (Sullivan & Winkelstein, 1994).

Infections are increased in C2-deficient individuals, as would be expected, and the most common cause of death among C2-deficient patients is sepsis (Jonsson et al., 2005). Other systemic infections such as meningitis, pneumonia, epiglottitis, and peritonitis have been seen, and the most common organisms have been *S. pneumoniae* and *H. influenzae* (Figueroa & Densen, 1991; Ross & Densen, 1984). With improved vaccination against these organisms, it is possible that the infection pattern will be altered.

C3 DEFICIENCY

C3 deficiency (MIM 120700) is the rarest of the four early component deficiencies, and it has the most severe phenotype by far (Botto et al., 1990, 1992; Grumach et al., 1988; Peleg et al., 1992; Singer et al., 1994). Membranoproliferative glomerulonephritis is seen in approximately a third of the cases of C3 deficiency, and SLE is rare (Figueroa & Densen, 1991; Ross & Densen, 1984). One specific C3 mutation has been associated with hemolytic uremic syndrome. All patients have a profound predisposition to infection, and the infections are sometimes characteristic of neutrophil dysfunction (abscesses), humoral deficiencies (sinopulmonary disease), and complement deficiencies (sepsis, meningitis). The severity of the infection phenotype dominates the clinical picture. Staphylococcal abscesses probably reflect an inability to opsonize and to generate the C5a chemotactic factor. Recurrent sinopulmonary infections reflect a significant compromise in B-cell costimulation. Systemic infections are due to a complete failure of C3b opsonization. These multiple defects contribute to the severe infectious manifestations of C3 deficiency (Reis et al., 2006).

One other feature of C3 deficiency deserves mention. During infections, a vasculitic rash may appear, and symptoms of serum sickness may occasionally be seen. These unusual findings are due to the lack of immune complex solubilization by C3. They are typically transient in nature but can cause confusion with lupus, particularly in the presence of glomerulonephritis.

C3 deficiency is rare, with fewer than 30 cases reported in the literature. There is a founder effect in South Africa among the Afrikaans-speaking population (Botto et al., 1992). The other mutations are diverse. Slightly more common is a partial deficiency of C3, termed hypomorphic C3 (McLean et al., 1978, 1985). This partial deficiency has been seen in a number of autoimmune disorders but is difficult to diagnose and is probably underascertained.

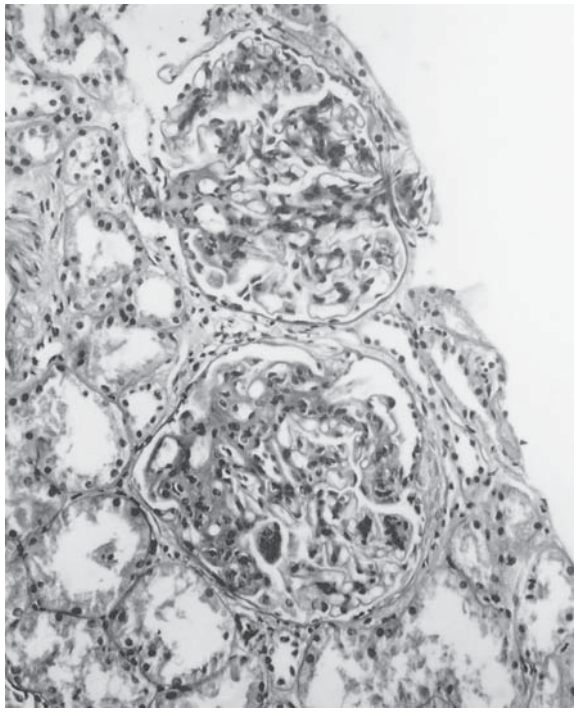

Figure 55.4 Glomerulonephritis in a patient with C4 deficiency. Inherited deficiencies of early complement components are associated with SLE and glomerulonephritis. This patient, with C4 deficiency, developed a malar rash and glomerulonephritis in early adolescence. A renal biopsy demonstrated increased mesangial matrix and cellularity. Immunofluorescence revealed deposits of IgG, C3, and C1q. (Courtesy of Thomas R. Welch, MD)

MBL DEFICIENCY

MBL deficiency (MIM 154545) was originally identified as associated with a variety of infectious diseases; however, it is quite common, with 2 to 7 percent of people having MBL deficiency (Thiel et al., 2006). Structural mutations and promoter mutations (Garred et al., 2006) both affect function. Because each mutation/polymorphism is relatively common, there are combinations of mild mutations that lead to complete loss of function.

A large number of studies have attempted to characterize the risk of infection in MBL-deficient individuals (Casanova & Abel, 2004; Thiel et al., 2006). It is felt to be more of a cofactor for infection in immune-compromised individuals than a direct cause of significant infection (Hansen et al., 2003; Sutherland et al., 2005).

MBL deficiency has also been examined as a risk factor for autoimmune diseases. It is associated with a risk of SLE (Garred et al., 2001), but in rheumatoid arthritis it is a modifier of disease, with the presence of MBL deficiency increasing radiological progression of disease (Graudal, 2004).

MASP2 DEFICIENCY

MASP2 deficiency (MIM 605102) was originally described in a single patient with autoimmune disease (Stengaard-

Pedersen et al., 2003). This patient suffered from ulcerative colitis and SLE. Laboratory evaluations revealed hypocomplementemia. Testing for MASP2 revealed very low levels, and a mutation was identified that interfered with MBL binding. It is now known that MASP2 deficiency occurs in 3.6 percent of Caucasians (Sorensen et al., 2005). MASP2 deficiency is frequently due to the D120G mutation, which abrogates its interaction with MBL (Thiel et al., 2007). Two mutations with founder effects among Africans and Amerindians have also been identified but are not as well characterized functionally (Lozano et al., 2005).

FACTOR B DEFICIENCY

Factor B deficiency (MIM 134350) was originally reported in association with neisserial infection (Densen et al., 1996b). The patient was identified after developing meningococcemia, and laboratory studies revealed an absent AH50. No mutation was characterized; however, abnormal protein was seen on electrophoresis. Several additional kindreds have been identified with gain-of-function mutations where the phenotype has been atypical hemolytic uremic syndrome (Goicoechea de Jorge et al., 2007).

FACTOR D DEFICIENCY

Neisserial infections are the most common manifestation of factor D deficiency (MIM 134350) (Figueroa & Densen, 1991; Kluin-Nelemans et al., 1984; Ross & Densen, 1984). Systemic streptococcal infections have also been seen (Weiss et al., 1998). Factor D is also known as adipsin, and mutations can also be associated with obesity (Flier et al., 1987). Other complement levels are typically normal in factor D deficiency; however, there is almost no ability to activate the alternative pathway.

PROPERDIN DEFICIENCY

Properdin deficiency (MIM 312060) is the only X-linked complement deficiency. It is one of the more common complement deficiencies and occurs largely in Caucasians. Approximately half of the properdin-deficient individuals have had one or more episodes of meningococcal disease (Densen et al., 1987; Figueroa & Densen, 1991; Fredrikson et al., 1996; Nielson & Koch, 1987; Ross & Densen, 1984; Sjoholm et al., 1982). Other bacterial infections are also seen but are much less common. There is a particularly high fatality rate in meningococcal disease in properdin-deficient patients, in contrast to the protection from mortality seen in patients with terminal complement component deficiencies. There may be a founder effect in Tunisian Jewish people; however, properdin deficiency is seen in subjects from all ethnic backgrounds.

In some families, properdin is undetectable, whereas in others affected males may have levels as high as 10 percent of normal. In still other kindreds, serum levels of antigenic properdin are normal but the protein is dysfunctional (Fijen et al., 1999). The serum of patients with properdin deficiency is unable to support the activation of C3 via the alternative pathway.

Mutations have been identified in several properdin-deficient patients. Three patients with no detectable serum properdin had mutations resulting in early stop codons (Fredrikson et al., 1996; Spath et al., 1997; Westberg et al., 1995). In kindreds in whom low levels of properdin were detected, different mutations were identified that resulted in amino acid substitutions (Westberg et al., 1995). One kindred with normal levels of dysfunctional properdin had a single base pair mutation in exon 9 resulting in the substitution of aspartic acid for tyrosine, rendering the molecule unable to interact with C3b (Fredrikson et al., 1996).

The role of properdin is to stabilize C3bBb, and in patients lacking this stabilizing function, activation of the alternative pathway is impaired. It is not completely clear why neisserial species are more subject to complement-mediated lysis than other gram-negative species. In vitro, most gram-negative organisms are susceptible to complement-mediated lysis, and it has been suggested that neisserial species are unusual in that they typically induce colonization of the nasopharynx prior to active infection. It may be that the site of inoculation is important, and other gram-negative organisms would be encountered predominantly in the gastrointestinal tract, where IgA is the dominant antibody and complement is not a prominent form of host defense.

C5 DEFICIENCY

C5-deficient patients (MIM 120900) have the same phenotype as the other terminal component deficiencies even though C5a is the major endogenous chemotactic factor for neutrophils (Figueroa & Densen, 1991; Ross & Densen, 1984; Wang et al., 1995). C5 deficiency is found in persons from a variety of ethnic and racial backgrounds. The mutations identified have included nonsense mutations and splice defects (Pfarr et al., 2005; Wang et al., 1995).

C6 DEFICIENCY

C6 deficiency (MIM 217050) is one of the more common complement disorders and occurs more frequently in African Americans and in people from South Africa. As is true for the other terminal component deficiencies, C6 deficiency is associated with meningococcemia, meningococcal meningitis, and disseminated gonococcal disease (Figueroa & Densen, 1991; Nishizaka et al., 1996a; Ross & Densen, 1984; Zhu et al., 1996). There are occasional reports of C6 deficiency associated with autoimmune disease, but it is likely that this represents ascertainment bias.

The most common form of C6 deficiency is characterized by undetectable serum levels of C6 and absent serum bactericidal activity. The basis for complete C6 deficiency in African Americans has been determined (Nishizaka et al., 1996a; Zhu et al., 1996). A homozygous single base pair deletion in exon

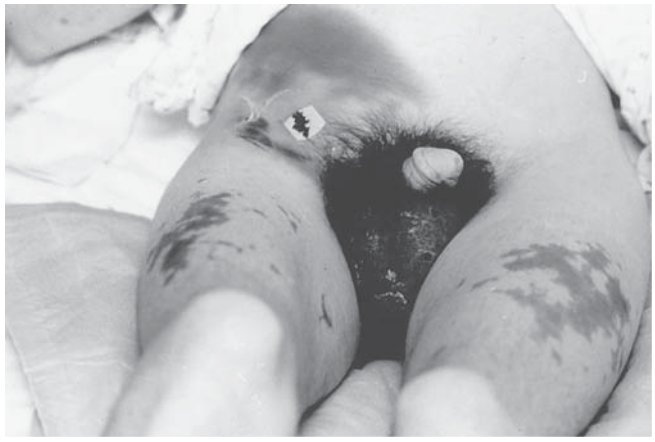

Figure 55.5 Meningococcal sepsis in a patient with C8 deficiency. Inherited deficiencies of terminal complement components are associated with an increased risk of meningococcal disease. This patient with C8 deficiency developed meningococcal meningitis and sepsis. (Courtesy of Peter Densen, MD)

12 that results in a frameshift and premature termination was found. Additional mutations have been identified and are diverse. Complete C6 deficiency occurs most frequently in South Africa, where haplotype determinations of flanking genes have demonstrated that the majority of C6 null alleles in this population arose from the same distant ancestor (Fernie et al., 1995, 1996b). However, in a minority of cases, the C6 null allele was found on a different haplotype, suggesting that there is some diversity even in this population. These haplotype determinations have been used to identify carriers in individual families.

There are two variations on C6 deficiency. In one case, a splice defect leads to a smaller-than-usual protein, C6SD (Wurzner et al., 1995). This protein functions less efficiently than wild-type C6; however, it is not clear whether bearing C6SD leads to compromised host defense. The other variation is combined C6 and C7 deficiency (Fernie et al., 1996a, 1996b).

C7 DEFICIENCY

C7 deficiency (MIM 217070) is not particularly common, and the few patients reported in the literature have had varied presentations. The most common presentation was neisserial disease (Figueroa & Densen, 1991; Nishizaka et al., 1996b; Ross & Densen, 1984). C7 deficiency can been seen in association with C6 deficiency, and the mutation is due to an arginine-to-serine change (Fernie et al., 1996b).

C8 DEFICIENCY

C8 is composed of three chains: α, β, and γ. The α and γ chains become covalently attached during synthesis and bind to the β chain. Interestingly, it is the α and β chain genes that are in linkage disequilibrium and map to chromosome 1p32. C8β deficiency (MIM 120960) is more common in Caucasians, while C8α-γ deficiency (MIM 120950, 120930) is more common among African Americans (Kaufmann et al., 1993; Kojima et al., 1998; Kotnik et al., 1997). The majority of the C8β mutations are due to a single base pair transition leading to a premature stop codon (Bellavia et al., 1996). The majority of the C8α-γ mutations are due to a 10 bp insertion leading to a stop codon (Densen et al., 1996a).

Regardless of the genetic basis, all types of C8 deficiency are associated with diminished bactericidal activity in vitro and an in vivo susceptibility to neisserial disease (Fig. 55.5) (Figueroa & Densen, 1991; Ross & Densen, 1984). Meningococcal meningitis, meningococcemia, and disseminated gonococcus have been seen. Rarely, C8 deficiency has been identified in patients with autoimmune disorders. The relationship of C8 deficiency to autoimmunity is not certain.

C9 DEFICIENCY

C9 deficiency (MIM 120940) is seen with high frequency in Asia (Hayama et al., 1989; Kang et al., 2005; Kira et al., 1999). Upwards of 0.05 percent of the population in Japan are C9-deficient. It is less frequent outside of Japan; however, it is also more difficult to diagnose than most of the other complement deficiencies described here because the CH50 is diminished but not absent (Hobart et al., 1997). C9-deficient serum can support some lytic activity, and the CH50 is typically one third to one half of normal in patients with C9 deficiency. This CH50 level would not typically lead to further evaluation. As is true for the other terminal complement component deficiencies, C9 deficiency is associated with neisserial disease, although the penetrance appears to be less than that seen in other terminal component deficiencies (Fukumori et al., 1989).

C1 INHIBITOR DEFICIENCY

Hereditary angioedema is due to heterozygous deficiencies of C1 inhibitor (C1INH). Infections and SLE are more common in this population but are subsidiary to the angioedema phenotype. The angioedema has no distinguishing features. It is not associated with urticaria, although a lacey reticular rash often precedes the onset of angioedema (Agostoni et al., 2004). The historical features most helpful in identifying potential C1 inhibitor-deficient individuals are recurrent episodes of angioedema, involvement of the airway in the absence of anaphylaxis, abdominal episodes, a positive family history, or angioedema arising after trauma (Agostoni et al., 2004).

There are two forms of C1INH deficiency (MIM 106100), both inherited as autosomal dominant traits (Rosen et al., 1965). In the more common form (type I), accounting for approximately 85 percent of patients, the serum of affected individuals is deficient in both C1INH protein (5 to 30 percent of normal) and C1INH functional activity (Cicardi et al., 1982; Frank et al., 1976; Rosen et al., 1971). The lower-than-expected levels of C1INH functional activity in heterozygotes are thought to be the result of increased catabolism of the normal protein (Ernst et al., 1996). In the less common form (type II), a dysfunctional protein is present in normal

or elevated concentrations, but the functional activity of C1INH is markedly reduced (Cicardi et al., 1982; Donaldson et al., 1985). In patients with type I C1INH deficiency, the diagnosis can be established easily by demonstrating a decrease in serum C1INH protein when assessed by immunochemical techniques. However, in patients with type II C1INH deficiency, the diagnosis must rest on demonstrating a decrease in C1INH functional activity. In either case, C4 levels are usually reduced below the lower limit of normal both during and between attacks (Austen & Sheffer, 1965; Pickering et al., 1968) because of their uncontrolled cleavage by C1s, thus making their measurement useful as a diagnostic clue.

This division of C1INH-deficient patients into two forms has no correlation with clinical phenotype and only limited correlation with genotype, as there are some examples of C1INH gene mutations in which a dysfunctional protein is produced but is degraded so rapidly that it is not detectable in the serum (Agostoni & Cicardi, 1992; Davis et al., 1993). Alu-mediated deletions and duplications account for the most common genotypes (Davis et al., 1993; Stoppa-Lyonnet et al., 1991). Four of the seven introns in the C1INH gene (C1NH) contain a total of 17 Alu-repetitive DNA elements. These Alu elements are responsible for most of the large deletions and duplications leading to C1INH deficiency. A variety of single base changes and smaller deletions and duplications have also been identified. Most patients with detectable but dysfunctional C1INH have a mutation that replaces the reactive center arginine residue. The other mutations identified in patients with dysfunctional protein are found in the hinge region and result in blockade of the reactive center loop, thereby inactivating the C1INH (Davis et al., 1993; Verpy et al., 1995).

The main manifestations of C1 inhibitor deficiency are recurrent episodes of submucosal or subcutaneous edema, although 5 percent of people who carry a C1 inhibitor mutation are asymptomatic (Agostoni et al., 2004). Half of patients have had episodes before the age of 10 years. The episodes may be as infrequent as one per year or as frequent as one per month, and the frequency and the severity of episodes do not correlate with laboratory features and are often inconsistent within a family. The extremities, face, and genitalia are most often involved (Color Plate 55.I). Involvement of the gastrointestinal tract can lead to disabling abdominal pain. Abdominal episodes begin with pain and are often accompanied by vomiting, or more rarely, diarrhea. Nearly three quarters of patients have this distressing feature. In one study, one third of patients with C1 inhibitor deficiency had undergone an appendectomy or exploratory laparotomy for abdominal pain (Agostoni et al., 2004). The most feared type of angioedema is that involving the airway. Although the lungs are not involved, the upper airway can swell, leading to respiratory arrest. This complication can occur in as many as two thirds of patients with C1 inhibitor deficiency, although improved management has made it somewhat less common. Prior to modern management, slightly over 10 percent of patients underwent a tracheostomy as a result of airway episodes.

The angioedema typically progresses for 1 to 2 days and resolves in another 2 to 3 days. Common precipitants are illness, hormonal fluctuations, trauma, and stress. Although many patients can identify triggers, many episodes have no identifiable trigger, which increases anxiety and feelings of loss of control. The C1 inhibitor promoter is androgen-responsive, which is why men have fewer problems, in general, than female patients (Falus et al., 1990; Lener et al., 1998; Prada et al., 1998). It may also explain the common complaint that symptoms vary with menstruation. The main therapeutic modality in the United States is based on the androgen responsiveness of the promoter and serves to increase expression of the intact gene.

The mechanism of angioedema is due to the production of bradykinin through the coagulation pathway. Hageman factor, also known as factor XII of the coagulation system, is activated by trauma-induced exposure of collagen on capillaries. C1INH inhibits the ability of activated Hageman factor to initiate kinin generation, and it inhibits kallikrein activation of the kinin system directly. Blister fluids from C1INH-deficient patients contain active plasma kallikrein, and their serum has decreased amounts of prekallikrein and kininogen (Curd et al., 1980), which suggests activation of the kinin system during attacks of edema. Furthermore, treatment with drugs that inhibit the release of vasoactive mediators is effective (Frank et al., 1972; Schneider et al., 2007; Sheffer et al., 1977) (Fig. 55.6).

C4 BINDING PROTEIN DEFICIENCY

C4 binding protein consists of multiple alpha chains and a single beta chain. It acts as a cofactor for factor I-induced cleavage of C4b and C3b. A single kindred with C4 binding protein deficiency (MIM 120830 120831), manifested by altered complement function, has been identified (Trapp et al., 1987). The proband presented with angioedema, vasculitis, and arthritis. The manifestations were thought to relate to uncontrolled activation of the classical pathway and release of anaphylatoxins. C4 binding protein interacts with protein S, and certain polymorphisms of C4 binding protein are associated with an increased risk of thrombosis.

FACTOR I DEFICIENCY

Factor I consists of two disulfide-linked polypeptides that act to regulate the alternative pathway-cleaving enzyme (C3b, Bb). One polypeptide carries serine protease activity that cleaves C3b to produce iC3b (inactive C3b), a C3-cleavage product that cannot function in the C3-cleaving enzyme of the alternative pathway.

Factor I deficiency (MIM 217030) has two phenotypes, and it is not yet clear whether a genotype–phenotype relationship exists. The first phenotype described relates to the role of factor I as a cofactor for C3bBb dissociation. When factor I is lacking, C3bBb continues to cleave C3 unabated and a secondary deficit in C3 occurs. Both the CH50 and AH50 are depressed, but not absent, and C3 antigen levels are low (Bonnin et al., 1993; Leitao et al., 1997; Vyse et al., 1994, 1996). The infectious consequences of low C3 are similar to those seen in true C3 deficiency (Figueroa & Densen, 1991; Ross & Densen, 1984). Neisserial disease has been seen as well as infections with more typical organisms such as

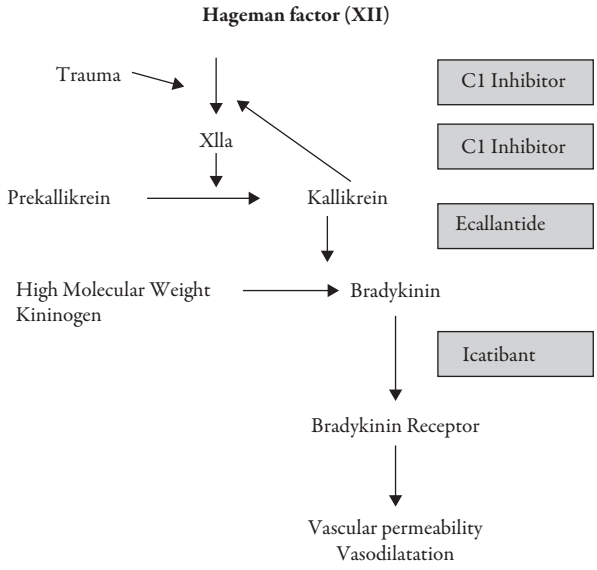

Figure 55.6 Hageman factor (factor XII) is predominantly activated by exposed collagen on damaged vascular tissue. Activated Hageman factor can cleave prekallikrein into active kallikrein. Kallikrein is part of a feed-forward loop, which leads to additional activation of Hageman factor and also cleaves high-molecular-weight kininogen into bradykinin. The gray boxes indicate the steps at which the various medications act.

S. pneumoniae and *H. influenzae*. As is true for inherited C3 deficiency, some patients have developed a serum sickness-like picture.

The second phenotype is atypical hemolytic uremic syndrome or membranoproliferative glomerulonephritis II (Fremeaux-Bacchi et al., 2004; Genel et al., 2005; Kavanagh et al., 2005). Hemolytic uremic syndrome is characterized by microangiopathic hemolytic anemia, renal disease, and hypertension. Atypical hemolytic uremic syndrome cases lack the common trigger of infectious diarrhea. Factor I deficiency is difficult to identify because complement studies are often normal. C3 levels may be depressed but are not necessarily depressed. The factor I level is typically normal as the mutations are not null. The mutations adversely affect binding to C3b and endothelium. The fenestrated endothelium of the glomerulus represents a landscape of polyanions where the basement membrane is exposed by the fenestration. Thus, the role of complement regulatory proteins would be greatest on those surfaces. Lack of protection due to lack of complement regulatory proteins would lead to endothelial damage in the glomerulus and hence hemolytic uremic syndrome.

FACTOR H DEFICIENCY

The gene for factor H (MIM 134370) lies within the RCA gene cluster on chromosome 1 (Holers et al., 1985). Factor H is synthesized in hepatocytes, macrophages, B cells, endothelial cells, and platelets. Factor H, like factor I, is an inhibitor of the alternative pathway. It competes with factor B for binding to C3b in the assembly of the alternative pathway C3-cleaving enzyme, C3bBb, and also displaces Bb once the enzyme has been assembled.

Infections, atypical hemolytic uremic syndrome, and macular degeneration are the main phenotypes seen in factor H deficiency (Dragon-Durey et al., 2004; Klein et al., 2005; Nielsen et al., 1989a; Sanchez-Corral et al., 2002). The infections are due to consumption of C3 and a secondary partial C3 deficiency (Reis et al., 2006). These can be easily suspected on the basis of diminished C3 levels and low but not absent CH50 and AH50, and typically the antigenic levels of factor H are low. Membranoproliferative glomerulonephritis has been identified in factor H deficiency, but the most common phenotype is atypical hemolytic uremic syndrome. Factor H deficiency was found to be the underlying basis for 15 to 30 percent of patients with atypical hemolytic uremic syndrome (Richards et al., 2007). Both autosomal recessive and heterozygous mutations have been seen. The age of onset is quite young in most cases, and the disease is recurrent (Caprioli et al., 2005). Death is not uncommon. These patients often have diminished C3 levels, although the antigenic level of factor H is typically normal or elevated. Because of the inconsistent complement laboratory analyses, mutation analysis is required for diagnosis.

A common tyrosine-to-histidine polymorphism of factor H was identified as a significant risk factor for macular degeneration in a genome-wide association study (Haines et al., 2005; Klein et al., 2005). Homozygous bearers of this polymorphic variant have a relative risk of 7.4 for the development of macular degeneration. Drusen are the characteristic retinal deposits seen in macular degeneration. These deposits contain factor H and terminal complement components. It has been hypothesized that the abnormal factor H provides less protection to the choroidal vessels, allowing smoldering complement activation and gradual damage to the endothelium.

MEMBRANE COFACTOR PROTEIN (CD46) DEFICIENCY

Deficiencies of membrane cofactor protein (MCP) are associated with a later onset of atypical hemolytic uremic syndrome compared to factor H and factor I deficiencies (Caprioli et al., 2005, 2006; Richards et al., 2003; Zimmerhackl et al., 2006). MCP mutations (MIM 120920) are thought to account for approximately 10 percent of all atypical hemolytic uremic syndrome cases. There is no other known phenotype for MCP deficiency. As MCP is a membrane protein, this defect is intrinsic to the kidney. In contrast to factor H and factor I deficiencies, renal transplantation can be successful. Traditional complement analyses are normal.

CD59 DEFICIENCY

A single patient with CD59 deficiency (MIM 107271) has been described, and the major manifestation was chronic hemolytic anemia (Yamashina et al., 1990). CD59 is expressed on most hematopoietic cells and endothelial cells, where it confers protection from intravascular complement-mediated lysis. This defect in CD59 was suspected because of the phenotypic resemblance to paroxysmal nocturnal hemoglobinuria (PNH).

DAF DEFICIENCY (CD55)

DAF deficiency (MIM 125240) is also termed the Inab blood group phenotype (Hue-Roye et al., 2005; Reid, 1990; Telen & Green, 1989). The Cromer blood group antigens reside on DAF, and the null phenotype is referred to as the Inab phenotype. Certain kindreds with DAF deficiency have had protein-losing enteropathy, while others have been completely healthy and have been identified at the time of blood donation or crossmatching for a transfusion. None of the patients have had hemolysis, suggesting that CD59 is substantially more important in regulating red cell lysis by complement.

CR3/CR4 DEFICIENCY

This disorder is a defect in the three β2 integrin adhesion molecules. It will be discussed in more detail in Chapter 53. The more common terminology for this β2 integrin deficiency is leukocyte adhesion deficiency type I (LAD 1). Mutations in the common β chain (CD18) (MIM 600065) lead to failure to express adequate α chains: CD11a, CD11b, and CD11c (Arnaout et al., 1990; Kishimoto et al., 1987). These three proteins are also known as LFA-1, CR3, and CR4.

The manifestations of the disorder are due to the combined effects of ineffective opsonization and an inability to migrate from the vascular space into the tissue space. β2 integrins are essential for the firm adhesion step and diapedesis. Lacking β2 integrins, the neutrophils remain in the vascular space, where they are unable to participate in the defense against bacteria.

DIAGNOSIS OF COMPLEMENT DEFICIENCIES

Most of the deficiencies of the classical pathway (C1 through C9) can be detected by using a hemolytic assay that assesses the functional integrity of the pathway. The most common of these, the CH50 assay, determines the ability of the patient's serum to lyse antibody-sensitized sheep erythrocytes. Since lysis depends on the functional integrity of the complete cascade, patients who have severe deficiencies of any of the individual components of the classical pathway will usually have less than 5 percent of normal total serum hemolytic activity. One exception is C9 deficiency. Because lysis of antibody-sensitized erythrocytes can occur in the absence of C9 (Stolfi, 1968), albeit to a lesser degree, patients with C9 deficiency have some total serum hemolytic activity, but usually between 30 and 50 percent of the normal level.

Deficiencies of components of the alternative pathway (factors B, D, H, I, and properdin) can be detected by using a hemolytic assay that tests the functional activity of the alternative pathway (AH50). The most common of these use rabbit erythrocytes, which are potent activators of the human alternative pathway (Polhill et al., 1978). Thus, patients with deficiencies of components of the alternative pathway, as well as patients with deficiencies of C5 to C9, will fail to lyse rabbit erythrocytes. Some factor H and factor I mutations do not affect the lytic ability in this assay. These are typically associated with atypical hemolytic uremic syndrome, and mutation testing is required for diagnosis in these cases.

Identification of the specific component that is deficient usually depends on both functional and immunochemical tests. Highly specific functional assays are available for each component. In each case, they depend on reagents that lack the specific component in question but provide the other components of the appropriate hemolytic pathway in excess. Monospecific antibodies are also available for each of the components and can be used in a variety of immunochemical assays to assess the presence or absence of the component in question. In most instances, the component will be deficient when assessed by both functional and immunochemical methods. However, there are some exceptions. Therefore, it is important to assess both antigen levels and functional levels when the index of suspicion is high.

MANAGEMENT OF COMPLEMENT DEFICIENCIES

Management of complement deficiencies requires specifically identifying the affected protein. For example, in inherited forms of atypical hemolytic uremic syndrome, renal transplants are indicated for MCP deficiency but not factor H or factor I deficiency. There are few genotype–phenotype correlations, and the main purpose of genetic testing for complement deficiencies is to confirm the defect. Where accurate protein identification is available, mutation testing is not required. The suggested strategies represent possible interventions based on the current literature. There are few clinical trials supporting a specific management strategy.

EARLY CLASSICAL COMPONENT DEFICIENCIES

The major features of early classical component deficiencies are SLE and infection. Patients with SLE are usually treated using standard modalities. One European study of C2-deficient SLE patients found replacement of C2 to be therapeutically useful; however, this product is not widely available (Erlendsson et al., 1993; Steinsson et al., 1989).

Therapy for infection is controversial, however. Patients are often given vaccines to raise the titers of antibodies to encapsulated organisms to high levels. In the case of terminal component deficiencies, high levels of antibody have been shown to partially compensate for the complement deficiency. For early complement component deficiencies, the major risks seem to be *S. pneumoniae* and *H. influenzae* (Figueroa & Densen, 1991; Jonsson et al., 2005; Ross & Densen, 1984). Vaccines to these entities exist, and there are theoretical reasons to believe that having high titers of antibody may offer protection. Prophylactic antibiotics are also often used to prevent life-threatening infections in patients. In one study, half of the C2-deficient patients had serious infections such as sepsis, and infection was the leading cause of death, accounting for over 10 percent of the deaths in this cohort (Jonsson et al., 2005). The range of age of deaths was quite broad, suggesting that

antibiotic prophylaxis might be required lifelong. Meningitis was also commonly seen. Thus, prevention of infection is desirable, and vaccination and prophylactic antibiotics should be given consideration. Patients taking immunosuppressive medication for rheumatological disorders may require more intensive efforts to prevent infection.

C3 DEFICIENCY

The optimal therapy for C3 deficiency is unknown. Infections in these patients are the most severe of any of the complement deficiencies, and management must address loss of opsonization, loss of B-cell costimulation, and loss of immune complex solubilization (Reis et al., 2006). One could consider the use of immune globulin replacement to compensate for the compromised B-cell function, and prophylactic antibiotic use could ameliorate some of the infections. Renal transplantation has been used in the setting of renal failure due to membranoproliferative glomerulonephritis. There is some recurrence risk. Nevertheless, as not all C3-deficient patients develop membranoproliferative glomerulonephritis, renal transplantation should be given consideration in patients with end-stage renal disease. Plasma infusions and liver transplantation as interventions remain theoretical options.

MBL DEFICIENCY

While MBL deficiency is common, infections arising in patients with MBL deficiency are not common (Garred et al., 2006; Thiel et al., 2006). Of those with infection, cofactors should be sought and treated. In the absence of treatable cofactors, antibiotics may be considered.

FACTOR D AND PROPERDIN DEFICIENCY

Patients with factor D and properdin deficiency have secondary consumption of C3. Neisserial disease is common and infections with *S. pneumoniae* and *H. influenzae* are also seen. Vaccination to achieve high titers of antibody to those entities could theoretically provide benefit. Traditionally, prophylactic antibiotics have been used for some patients in an effort to prevent infections (Biesma et al., 2001; Hiemstra et al., 1989; Sjoholm et al., 1988).

TERMINAL COMPLEMENT COMPONENT DEFICIENCIES

Deficiencies of C5, C6, C7, C8, and C9 are all associated with an increased risk of neisserial disease. Meningococcal disease is by far the most common, but disseminated gonococcal infections have been seen with significant frequency, and patients should be warned about the possibility. The prevention of meningococcal disease has been studied thoroughly in Russia and Europe, and two things have emerged from these studies. Patients with terminal complement component deficiencies have a rather abrupt onset of meningococcal disease but have a shorter and milder course ultimately (Platonov et al., 1993). Thus, patients in rural areas may be at increased risk due to potential delay in the initiation of treatment. The other important lesson is that vaccination every 3 years with the meningococcal vaccine decreases the frequency of meningococcal episodes but does not eliminate them (Drogari-Apiranthitou et al., 2000; Fijen et al., 1998; Platonov et al., 1995; Schlesinger et al., 2000). The frequency is decreased to 20 percent of what nonvaccinated individuals experience. No study has examined prophylactic antibiotics, and it may be that careful monitoring and hyper-vaccination is sufficient.

C1 INHIBITOR DEFICIENCY

Treatment of C1INH deficiency is different from that of other complement deficiencies in that there are specific measures available to ameliorate symptoms and prevent recurrences. In many patients, episodes of angioedema may occur frequently enough or be so difficult to manage as to justify long-term prophylaxis. Attenuated androgens such as oxandrolone or danazol are highly effective (Gelfand et al., 1976; Gompels et al., 2005) and act by increasing transcription of the normal allele of C1INH (Agostoni et al., 1980b). Another class of agents used for long-term prophylaxis are the antifibrinolytic agents (Frank et al., 1972; Sheffer et al., 1977; Van Dellen, 1996). Tranexamic acid and aminocaproic acid act by blocking plasmin generation. Although their efficacy is less than that of attenuated androgens, the incidence of side effects is also less than that of attenuated androgens. Antifibrinolytics may have usefulness in childhood but are difficult to obtain and have been largely supplanted by other modalities. Long-term prophylaxis with C1 inhibitor is available but is extremely expensive and requires twice-weekly intravenous infusions. For patients with frequent episodes, it represents a significant advance in normalizing their lives.

In some instances, patients may require short-term prophylaxis for surgery or oral procedures. Attenuated androgens, fibrinolytic agents, fresh-frozen plasma (FFP), and C1 inhibitor concentrate have all been used successfully for short-term prophylaxis. C1 inhibitor concentrate would be considered the most effective approach at this time.

Acute attacks can be life-threatening and can require urgent, aggressive intervention. C1 esterase inhibitor concentrate, bradykinin B2 receptor antagonist, and a kallikrein inhibitor are options (Agostoni et al., 1980a; Waytes et al., 1996). Each has advantages, with the currently approved products requiring a medical support system except for the bradykinin B2 receptor antagonist, which can be self-administered. Supportive care and close observation are essential for pharyngeal swelling because it can progress to airway compromise in a few hours (Figure 55.7 and Color Plate 55.I). Narcotics are appropriate for abdominal pain episodes. When the more modern agents are not available, antifibrinolytics are thought to reduce the severity and length of the episode, and attenuated androgens may do the same; however, they do not begin to have an effect for 24 hours. At various times, FFP and aprotinin have been used for acute episodes; however, FFP is thought to provide active substrate to enhance further edema and is not routinely used, and side effects with aprotinin have

limited its use. Epinephrine, antihistamines, and corticosteroids are of no proven benefit in C1INH-deficient patients.

Medical interventions for short-term prophylaxis, long-term prophylaxis, and acute therapy are given in Table 55.6. There are a number of strategies that should be reviewed with each patient (Agostoni et al., 2004). Genetic counseling should be offered to each family. The use of bracelets with medical information is an individual choice. For patients with anticipated travel or events that might lead to isolation from friends and family familiar with the disorder, bracelets might have some role to inform paramedics and physicians in the event of an emergency. Patients should be counseled to avoid certain medications. Angiotensin-converting enzyme (ACE) inhibitors can induce angioedema, as can estrogen-containing birth control pills or postmenopausal hormone replacement. In men, hormonal modulation may also affect their angioedema.

The last category of management is fertility and obstetrical management. Polycystic ovary syndrome is seen in approximately one third of female patients with C1 inhibitor deficiency regardless of prior therapy (Agostoni et al., 2004). The typical endocrine findings of increased luteinizing hormone and testosterone are not seen. Ultrasounds demonstrate polycystic ovaries, and the only laboratory feature is often reduced levels of follicle stimulating hormone. Menstrual irregularities are common, and the underlying pathogenesis is the aberrant regulation of complement activation in follicular fluid (Perricone et al., 2000).

Despite common menstrual irregularities, fertility is largely preserved, and pregnancy poses a particular risk to

Table 55.6 **THERAPEUTIC OPTIONS FOR C1 INHIBITOR DEFICIENCY***

TREATMENT	ADULT	PEDIATRIC	COMMENTS
Tranexamic acid (Cyclokapron)	1–3 g/d PO as divided doses for prophylaxis, 1 g po q3–4h until episode resolves for acute episodes	25–50 mg/kg BID or TID as prophylaxis, 1.5 g/d for acute episodes (available as IV form)	Not available in the USA
Epsilon aminocaproic acid (Amicar)	1 g po TID as prophylaxis, 1 g/h as IV therapy for acute attacks	100 mg/kg q4–6h not to exceed 30 g/d as therapy. Oral syrup available for prophylaxis but doses not established: 6 g/d for children <11 y and 12 g/d for children >11 y has been used successfully.	The only antifibrinolytic available in the USA. Modest efficacy. Cannot be used in neonates. Oral dosing has significant GI side effects.
Danazol (Danocrine)	200 mg po QD as a starting point for prophylaxis (titrate to effect), 400–600 mg po QD for acute episode or short-term prophylaxis	50–200 mg po QD as a starting point for prophylaxis; titrate to effect and consider QOD or Q 3 days in preadolescent children; can use up to 400 mg po QD as short-term prophylaxis	Concern about androgenization and premature closure of the epiphyses limits the use of attenuated androgens in children. Titration to desired effect is recommended rather than to laboratory criteria.
Oxandrolone (Oxandrin)	2.5–20 mg po TID as prophylaxis (titrate to effect). Not proven as short-term prophylaxis or treatment.	0.1 mg/kg/d as prophylaxis. Not proven as short-term prophylaxis or treatment in a formal clinical trial.	Has fewer androgenizing effects than danazol
Fresh-frozen plasma (FFP)	2 U IV as short-term prophylaxis. May be required for up to 36 hours after surgery.	10–15 mL/kg as short-term prophylaxis. May be required for up to 36 hours after surgery.	Not typically used for acute episodes due to danger of accelerating angioedema; inexpensive for short-term prophylaxis for surgery or dental extractions
C1 inhibitor concentrate (Cinryze, Berinert)	20 U/kg as IV treatment using Berinert, 1,000 U IV every 3–4 days for prophylaxis using Cinryze	10–30 U/kg as treatment (up to 500–1,000 U total)	Very rapid effect; especially useful in pregnancy; self-infusion possible with training
Ecallantide (Kalbitor)	10 mg subQ ×3	Not FDA approved for children	High rate of anaphylaxis; requires medical supervision
Icatibant (Firazyr)	30 mg subQ into abdomen for acute episodes	Not FDA approved for children. Injection site pain may limit use in young children.	Bradykinin receptor antagonist. Cannot be used with angiotensin-converting enzyme (ACE) inhibitors.

* Supportive care:

Airway protection, fluid replacement, and pain relief are of paramount importance. Some episodes require no intervention. For long-term prophylaxis, consider monitoring liver by ultrasound and blood studies. Although epinephrine is ineffective systemically, it may provide some benefit when used topically in airway obstruction.

both the mother and the fetus. The hormonal shifts of pregnancy lead to an increased risk of angioedema, although late pregnancy seems to offer some protection. Delivery is itself traumatic, and an affected mother has a 50 percent chance of transmitting the disorder to her offspring. Thus, potentially both mother and child are at risk during delivery. There is no consensus on management. C1 inhibitor may be given prophylactically or if problems arise.

FACTOR H, FACTOR I, FACTOR B, AND MCP DEFICIENCY

It is thought that certain mutations in factor H, factor I, and factor B deficiency predispose to meningococcal disease while others predispose to hemolytic uremic syndrome. In kindreds with meningococcal disease, the same strategies utilized for patients with terminal complement component deficiencies would be expected to be of benefit. In kindreds with hemolytic uremic syndrome, the management is less clear. As is done for thrombotic thrombocytopenic purpura (TTP), some patients receive pheresis and FFP replacement for acute episodes (Caprioli et al., 2006; Goodship, 2006). One study evaluating factor H replacement demonstrated benefit, suggesting that FFP alone might be of benefit as prophylaxis. In the case of MCP, where the affected protein is membrane-bound, it is less clear that pheresis and FFP would provide benefit, but it could potentially act to clear inciting agents or complement activation products. For patients with end-stage renal disease, the recurrence of disease in patients with factor H or factor I deficiency is unacceptably high and renal transplantation is not recommended. In contrast, renal disease in MCP typically does not recur in the transplanted kidney.

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56.

ASSESSMENT OF THE IMMUNE SYSTEM

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INTRODUCTION

In this chapter, we discuss the assessment of immune function in the context of a diagnostic approach to the patient with suspected primary immunodeficiency. We will begin by discussing general elements of the clinical history and physical findings in the broad spectrum of primary immunodeficiency diseases (PIDDs) and how these guide the choices of screening tests to be performed in the initial laboratory evaluation. We will then discuss more specific tests that may be used to further define functional deficits in an attempt to focus ultimately on one or a few molecular tests to establish (wherever possible) a definitive diagnosis at the molecular level. We strongly advocate diagnosis at the molecular level for the following reasons: (1) unequivocal diagnosis, (2) accurate genetic counseling, (3) planning future pregnancies or their outcomes, (4) definition of genotype–phenotype associations, and (5) identification of candidates for gene-specific therapies (Bonilla et al., 2005).

In our discussion, we will mainly follow the World Health Organization/International Union of Immunological Societies classification of PIDDs (Al-Herz et al., 2011). Our goal is to provide a practical guide for clinical and laboratory assessment. Rather than list specific diagnostic criteria or describe disease-specific testing for each PIDD, the reader is referred to each disease-specific chapter for this information.

The Internet affords additional sources of information accessible to the public and to health-care professionals. In many cases, this information is of high quality and current and can assist physicians with diagnosis and management problems. Table 56.1 lists several sources of information available on the Internet.

CLINICAL ASSESSMENT

AGE OF ONSET

It is a common misconception that all PIDDs have their onset in infancy or early childhood and will be so evident as not to escape detection. Although this is certainly true of many severe disorders, even among these, a failure to consider the possibility of immunodeficiency underlying a severe infection leads to undue morbidity and mortality. Mild or partial variants of many of the "severe" forms of PIDDs may more easily escape attention until later in childhood or even adulthood. Furthermore, it is important to appreciate the sometimes extreme (years to decades long) delay in diagnosis of PIDD patients after the onset of their recurrent infections and other complications. Finally, a significant fraction of these disorders will present initially in adulthood. See Wood et al. (2007) for a discussion in the general setting of antibody deficiency.

The only PIDD that characteristically presents at any age, including adulthood, is common variable immunodeficiency (CVID; see Chapter 28). Most other disorders have their onset in infancy or childhood. However, as already mentioned, they may escape detection for some time, or the patient may die without a diagnosis. There are abundant reports of mild cases of many forms of PIDD such as combined immunodeficiency, agammaglobulinemia, neutrophil defects, and complement component deficiencies that either have their clinical onset in adulthood or are not diagnosed until then. These are mainly due to so-called "hypomorphic" gene mutations that lead to only partial loss of function of the gene product and milder disease phenotype (see specific chapters for each PIDD). However, other genetic factors also play a role, and some individuals who have the same mutation will have severe disease

Table 56.1 SOURCES OF INFORMATION ON PIDDS ON THE INTERNET

URL	NAME/DESCRIPTION
http://www.aaaai.org	American Academy of Allergy, Asthma, and Immunology
http://www.esid.org	European Society for Immunodeficiencies
http://www.info4pi.org	Primary Immunodeficiency Resource Center
http://www.ipidnet.org	Immune Phenotyping in Primary Immunodeficiency
http://www.ipopi.org	International Patient Organization for Primary Immunodeficiencies
http://www.jmfworld.org	Jeffrey Modell Foundation
http://www.primaryimmune.org	Immune Deficiency Foundation*

*A consultation service is available for questions from physicians.

of early onset, while others will have mild late-onset disease, or may even be completely asymptomatic (Bonilla et al., 2005). X-linked agammaglobulinemia is a good example of this phenomenon (see Chapter 25). Table 56.2 lists the general pattern of age at presentation for the main categories of PIDD.

INFECTIONS

PIDD most commonly comes to clinical attention due to a burden of infection that is considered to be excessive with respect to frequency or severity, displays a poor response to therapy, or is associated with unusual organisms. The frequency of infection and the types of organisms found will vary widely depending on a variety of related factors. These include age (more infections at the extremes of young and old age; High, 2004; Ulloa-Gutierrez et al., 2003), socioeconomic status, day care or school attendance, breastfeeding, and number of children in the household (Simoes, 2003). Even lifestyle choices such as frequent exercise may be associated with reductions in acute upper respiratory infections (Matthews et al., 2002). Factors such as geographical location, public hygiene (Esrey et al., 1991), and other environmental exposures such as pollution (Ciencewicki & Jaspers, 2007) also have an impact.

Innate and adaptive immune mechanisms operate in concert in most infections regardless of the type of infecting agent (viruses, bacteria, mycobacteria, mycoplasma, fungi, protozoa). However, a molecular lesion in one element of either innate or adaptive immunity may lead to a relatively narrow or selective spectrum of infectious agents and a pattern of infection that is characteristic and may provide some basis for structuring the immune evaluation (Al-Herz et al., 2011; Bonilla et al., 2005). It is not practical to provide a complete list, as a tremendous variety of specific infections may occur in many of these diseases. Table 56.2 provides a general list of the types of infections predominantly or characteristically associated with the major classifications of PIDD.

AUTOIMMUNITY

Autoimmune disease is a frequent complication of many forms of PIDD. The most consistent associations are seen with CVID (Cunningham-Rundles, 2008; Lopes-da-Silva & Rizzo, 2008), Wiskott-Aldrich syndrome (WAS) (Ochs et al., 2008), and complement deficiency (Wen et al., 2004). However, autoimmunity is observed in many other syndromes and is the principal clinical problem in autoimmune lymphoproliferative syndrome (ALPS; Chapter 30) and the syndromes of autoimmunity, polyendocrinopathy, candidiasis, ectodermal dystrophy (APECED, Chapter 31) and immune dysregulation, polyendocrinopathy X-linked (IPEX, Chapter 32). Table 56.2 lists some general associations.

LYMPHOPROLIFERATION

Lymphoproliferative disease and lymphoma are also frequently seen in a number of PIDDs. Again, the most consistent associations are with CVID and WAS. Other syndromes with a high incidence are X-linked lymphoproliferative disease (XLP, Chapter 44), ALPS, and DNA breakage syndromes (Chapters 46 to 49). In CVID and ALPS, association with Epstein-Barr virus (EBV) is infrequent, while in WAS and XLP, EBV-association is common. Table 56.2 also lists PIDDs associated with lymphoproliferative disease.

ATOPY

IgE-mediated hypersensitivity may occur in any PIDD in which IgE responses are possible. However, it is seen with greater frequency in mild antibody deficiencies such as selective IgA deficiency (see Chapter 28), IgG subclass deficiency (Olinder-Nielsen et al., 2007), and specific antibody deficiency (Paris & Sorensen, 2007), as well as in some syndromes such as WAS (Chapter 43) and Netherton syndrome (Renner et al., 2009). In IPEX, IgE-mediated food allergy is essentially universal. However, in this disorder the enteropathy is due primarily to lymphocytic infiltration. In the hypomorphic severe combined immunodeficiency (SCID) variants grouped under the name Omenn syndrome (Chapter 13) and in hyper-IgE syndrome (Chapter 38), a very large amount of IgE is produced. Most of the IgE in these disorders is not specific for definable antigens, although occasional antigen-specific IgE is observed. Staphylococcal-binding IgE is often found in hyper-IgE syndrome. The main differential non-PIDD diagnoses of elevated IgE are parasitic infestation and atopy (especially atopic dermatitis, where staphylococcal-binding IgE is also frequently present).

Table 56.2 CLINICAL CHARACTERISTICS OF PIDDS

	CATEGORY OF PIDD					
CLINICAL CHARACTERISTIC	PREDOMINANTLY ANTIBODY DEFICIENCIES	CELLULAR DEFECTS	COMBINED DEFECTS	COMPLEMENT DEFICIENCIES	PHAGOCYTIC CELL DEFECTS	DEFECTS OF INNATE IMMUNITY
Age of onset	AGAM: infancy/ early childhood CVID: any age, often adulthood IGGSD, IGAD, SAD: usually childhood, occasionally adulthood	Usually infancy or early childhood	Usually infancy, rarely later	Often infancy/ childhood, but may have delayed presentation	Usually infancy/ childhood, occasionally adulthood	Usually infancy/ childhood
Infections	Respiratory tract viral and bacterial infections (mainly encapsulated organisms), other invasive bacterial infections, enteroviral infections, giardiasis	Mycobacterial infections, tuberculosis and atypical, salmonella	Opportunistic infections (PCP), disseminated infections with all categories of pathogens	Respiratory tract and other invasive bacterial infections (sepsis, meningitis), mainly with encapsulated organisms. Esp. *Neisseria* infection with terminal component defects.	Respiratory and deep-seated bacterial abscesses with staphylococci, also fungi (*Aspergillus*)	Extremely variable depending on specific defect
Autoimmune Disease	AGAM: rare CVID: common IGGSD, IGAD, SAD: common with IGAD, otherwise infrequent	Uncommon	Occasional	Frequent, esp. with early classical pathway defects	IBD is common in CGD, otherwise rare	Variable depending on defect
Lymphoproliferative disease	AGAM: rare CVID and IGAD: frequent, otherwise rare	Rare	Occasional	Rare	Rare	Uncommon
Atopy	AGAM/CVID: not seen, otherwise common	Uncommon	Variable, depends on defect	Similar to the general population	Similar to the general population	Uncommon

OTHER MANIFESTATIONS OF PIDD

Many PIDDs have "nonimmunological" associated features that may aid in guiding the evaluation and raising suspicion regarding specific diagnoses. Table 56.3 lists several examples of these types of clinical features along with some of the particular infectious complications associated with specific defects.

DIAGNOSTIC APPROACH

STEPWISE APPLICATION OF TESTING

In patients with suspected PIDD, laboratory evaluation usually proceeds through several stages (Bonilla et al., 2005). Initial testing is largely dictated by the type of deficit anticipated according to the clinical presentation (Tables 56.2 and 56.3). These screening tests attempt to establish a deficient area of immune function that can be studied with more refined or detailed tests in an attempt to achieve a definitive diagnosis (Table 56.4).

SUSPECTED ANTIBODY DEFICIENCY

Recurrent respiratory tract bacterial infections are one of the hallmarks of defects of antibody formation. Initial testing of humoral immunity consists of measurement of serum immunoglobulin levels and random antibody titers to vaccines. The measurement of IgG subclasses is controversial since there is no consensus regarding the clinical definition and significance of IgG subclass deficiency. If antibody levels for one or more vaccines are low, booster immunization and repeat measurement 4 weeks later should be undertaken wherever possible, unless IgG levels are so low as to make this untenable (<1 g/L). Significant hypogammaglobulinemia or impairment of specific antibody formation requires enumeration of peripheral blood B cells, usually performed as part of a panel quantifying T-cell subsets and natural killer (NK) cell

Table 56.3 CLINICAL FEATURES AND INFECTIOUS COMPLICATIONS ASSOCIATED WITH COME PIDDS

SYNDROME	LYMPHATIC	HEMATOLOGICAL	DERMATOLOGICAL	SKELETAL/DENTAL	RESPIRATORY	GASTROINTESTINAL	NEUROLOGICAL	ENDOCRINE	CARDIOVASCULAR
X-linked SCID	Aplasia of tonsils and thymus	Lymphopenia, eosinophilia	Candidiasis			Candidiasis	Encephalitis		
Autosomal recessive SCID due to defects of cytokine signaling	Aplasia of tonsils and thymus	Lymphopenia, eosinophilia	Candidiasis	Short stature (STAT-5b)			Encephalitis, myopathy (ORAI1 and STIM1 deficiencies)	Growth hormone insufficiency (STAT-5b)	
SCID due to RAG deficiency	Aplasia of tonsils and thymus	Lymphopenia, eosinophilia					Encephalitis		
Omenn syndrome	Splenomegaly, lymphadenopathy	Lymphopenia, eosinophilia	Eczema, alopecia			Hepatomegaly			
ADA deficiency		Lymphopenia, eosinophilia					Cognitive impairment		
PNP deficiency		Lymphopenia, AIHA					Cognitive impairment, ataxia		
MHC class I deficiency			Mucosal ulceration						Vasculitis
MHC class II deficiency			Candidiasis			Persistent diarrhea			
Hyper-IgM due to CD40 or CD40 ligand deficiency		Neutropenia, AIHA	Mucosal ulceration	Arthritis	Bronchiectasis	IBD, hepatomegaly, sclerosing cholangitis	Cryptococcal meningitis, toxoplasmosis		
Autosomal hyper-IgM syndromes	Lymphadenopathy, splenomegaly				Bronchiectasis				
XLA	Aplasia of tonsils				Bronchiectasis	Gastritis, Crohn's disease	Enteroviral encephalitis, myositis, bacterial meningitis, progressive neurodegeneration		
Autosomal recessive agammaglobulinemia	Aplasia of tonsils				Bronchiectasis	Gastritis		Growth hormone insufficiency (special variant)	

(continued)

Table 56.3 CONTINUED

SYNDROME	LYMPHATIC	HEMATOLOGICAL	DERMATOLOGICAL	SKELETAL/DENTAL	RESPIRATORY	GASTROINTESTINAL	NEUROLOGICAL	ENDOCRINE	CARDIOVASCULAR
CVID	Lymphadenopathy, splenomegaly	AIHA, AITP; neutropenia	Hypopigmentation	Arthritis	Interstitial lung disease, granuloma, bronchiectasis	Gastritis, IBD, celiac disease, nodular lymphoid hyperplasia, hepatomegaly, granuloma	Granulomatous disease (rare)	Autoimmune thyroiditis	
ALPS	Lymphadenopathy, splenomegaly	AIHA, ITP, neutropenia	Alopecia			Hepatomegaly			
APECED			Candidiasis, ectodermal dystrophy, alopecia	Enamel hypoplasia		Mucosal candidiasis		Hypothyroidism, hypoparathyroidism, Addison disease, type 1 diabetes, ovarian failure,	
IPEX	Lymphadenopathy	AIHA, neutropenia, ITP	Erythema, eczema, alopecia			Hepatosplenomegaly, enteritis, villous atrophy		Type 1 diabetes, autoimmune thyroiditis	
Periodic fever syndromes					Pleuritis	Peritonitis			
Defects of the IL-12/23-IFN-γ axis		*Salmonella* infections		Osteomyelitis due to atypical mycobacteria	Tuberculosis (often atypical mycobacteria)	Persistent *Salmonella* infections			
Innate immunodeficiencies			Skin abscess, impaired wound healing	Arthritis, osteomyelitis	Recurrent bacterial infections, tuberculosis		Herpes simplex encephalitis		
Ataxia-telangiectasia			Café-au-lait spots, hypopigmentation, telangiectasia		Bacterial infections		Cognitive impairment, ataxia		Telangiectasia
Wiskott-Aldrich syndrome		Thrombocytopenia, neutropenia, AIHA, eosinophilia	Eczema	Arthritis	Bacterial infections	Bloody diarrhea			Vasculitis
XLP	Splenomegaly, lymphoma	Anemia				Hepatitis	Vasculitis		
DiGeorge syndrome	Aplasia of thymus	Lymphopenia, AIHA	Erythema	Arthritis, micrognathia			Cognitive impairment	Hypoparathyroidism	Cardiac malformation

Hyper-IgE syndromes		Eosinophilia	Eczema, skin abscesses, candidiasis, warts (AR)	Joint hyperextensibility, fractures, persistence of deciduous teeth	Abscesses, pneumatoceles, bronchiectasis, aspergillosis				CNS vasculitis (AR)
Cartilage-hair hypoplasia		Neutropenia, anemia	Fine sparse hair	Short stature		Hirschsprung disease		Defective spermatogenesis	
Anhidrotic ectodermal dysplasia with ID			Fine sparse hair, anhydrosis	Conical teeth, osteopetrosis	Bronchiectasis	IBD, celiac disease			
CGD	Lymphadenopathy, splenomegaly	Eosinophilia	Skin abscess	Osteomyelitis	Granuloma, bronchiectasis, extrinsic allergic alveolitis	Gastritis, IBD, hepatomegaly, granuloma, liver abscess, aspergillosis	Aspergillosis		Cardiomyopathy
Leukocyte adhesion defects	Bleeding tendency (LAD3)	Leukocytosis	Mucosal ulceration, impaired wound healing	Periodontitis, microcephaly (LAD2)		Perityphlitis (LAD1)	Cognitive impairment (LAD2)		
Cyclic and congenital neutropenia		Neutropenia, eosinophilia	Mucosal ulceration, skin abscess	Periodontitis			Cognitive impairment (Kostmann s.), bacterial meningitis		
Chediak-Higashi syndrome	Splenomegaly	Bleeding tendency	Hypopigmentation				Seizures, cognitive impairment		
Familial hemophagocytic syndromes	Splenomegaly	Anemia, neutropenia, hemophagocytosis	Hypopigmentation (some)			Hepatomegaly, elevated liver enzymes	Cognitive impairment		CNS vasculitis
Complement deficiencies		Sepsis (late complement components)	Lupus- like erythema (early complement components)				Meningococcal meningitis		Vasculitis

ABBREVIATIONS: AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenic purpura; AR, autosomal recessive; CNS, central nervous system; IBD, inflammatory bowel disease; ID, immunodeficiency. See text for other abbreviations.

Table 56.4 SCREENING AND ADVANCED TESTS FOR EVALUATING IMMUNE SYSTEM FUNCTION

SYSTEM	SCREENING TESTS	ADVANCED TESTS
Innate immunity		
Humoral: complement	• CH50 (classical pathway activity) • AH50 (alternative pathway activity) • Lectin pathway activity	• Determination of level or function of individual components • Molecular methods
Cellular: phagocytic	• Cellular blood count with differential • Examination of a stained blood smear • Measurement of oxidase function (nitroblue tetrazolium test, or DHR dye reduction)	• Flow cytometry for adhesion molecules • Measurement of chemotaxis, phagocytosis, or intracellular killing • Enzyme assays (myeloperoxidase, G6PDH) • Bone marrow biopsy • Molecular methods
Cellular: NK cells	• Flow cytometry to enumerate NK cells • NK-cell function (spontaneous K562 lysis)	• ADCC • Surface marker expression • Molecular methods
Cellular: TLRs	• Cytokine production in response to TLR agonists	• Molecular methods
Adaptive immunity		
Humoral: antibody and B cells	• Serum immunoglobulin isotype levels • Specific antibody levels • Flow cytometry to enumerate B cells	• Antibody response to booster immunization • Antibody response to neoantigen • In vitro immunoglobulin production • Flow cytometry for surface and cytoplasmic markers • Molecular methods
Cellular: T cells	• Flow cytometry to enumerate T-cell subsets • Cutaneous delayed hypersensitivity • Mitogen/antigen proliferation assays • Anti-CD3 proliferation assay	• Flow cytometry for expression of surface markers (with/without activation) • Flow cytometry or other assays of cytokine production (with/without activation) • Flow cytometry for phosphorylation of signaling intermediates (e.g., pSTAT5b in X-linked SCID) • Cytotoxicity assay • Enzyme assay (ADA, PNP) • Molecular methods

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; G6PDH, glucose-6-phosphate dehydrogenase. See text for other abbreviations.

populations at the same time. Assessment of memory B-cell populations (class-switched and nonswitched) is becoming increasingly common. Abnormalities in immunoglobulin and/or antibody production may arise from defects intrinsic to B cells or to T-effector/helper cells; both compartments may require investigation, depending on the clinical presentation and results of screening tests.

Some complement defects may mimic clinically the disorders of antibody production (see Chapter 55). This is not surprising considering the role of complement in the opsonization of encapsulated bacteria coated with antibody. In the absence of complement, phagocytosis is less efficient.

SUSPECTED CELLULAR OR COMBINED IMMUNE DEFICIENCY

Initial testing for suspected cellular or combined immunodeficiency (CID) generally includes both tests of humoral immunity and tests of cellular immunity. Screening tests of the latter generally include measurement of lymphocyte subpopulations and a functional test such as cutaneous delayed hypersensitivity or in vitro lymphocyte proliferation tests. Early-onset severe immunodeficiency may indicate SCID or a SCID variant. Significantly diminished T-cell number and near-absent T-cell function will be present in the majority of cases of SCID, and directed molecular testing is dictated by the lymphocyte phenotype (see Chapter 9). SCID-variant phenotypes or other CID with early onset may be difficult to distinguish, and several rounds of molecular testing may be necessary based on the predicted likelihood of various defects consistent with the specific clinical and laboratory phenotype.

RECURRENT ABSCESSES

Skin and other organ abscesses caused by bacteria and fungi are characteristic of phagocytic cell defects. Screening tests of humoral and cellular immunity are often performed to establish that they are normal, but the main focus initially should be on determining neutrophil number and morphology. When these tests are normal, a test of oxidative function is appropriate to investigate the various forms of chronic granulomatous disease (CGD). In adults, when hyper-IgE syndrome has been excluded, an underlying defect responsible for recurrent abscesses can often not be determined with current methods.

LATE ONSET

PIDDs with late onset may represent CVID or hypomorphic variants of disorders that characteristically present earlier or may be due to late acquisition of the defining illness in those disorders with very restricted sensitivity to one or a few pathogens. It is important to consider acquired immunodeficiency due to HIV infection, malignancy (especially lymphoma), or secondary immunosuppression in these cases also.

LYMPHOPROLIFERATION

Lymphoproliferation leads to splenomegaly and lymphadenopathy, which should be assessed by appropriate imaging modalities (ultrasound or computed tomography for spleen and abdominal lymph nodes, chest X-ray for screening, and tomography for confirmation of thoracic involvement). A central concern in the diagnostic approach to lymphoproliferative disease is the exclusion of malignancy. Therefore, a biopsy should be undertaken whenever possible. There are no serologic or cellular markers in the peripheral blood that are sufficient to exclude malignant disease. Similarly, the observation of oligoclonal populations in the peripheral blood and tissue does not permit a definitive diagnosis of malignant transformation, since these changes have also been found in reactive lymph nodes (Sander et al., 1992). In addition, lymphoproliferation in PIDD might be secondary to infection, including EBV, and mycobacteria, including atypical organisms. Therefore, the consulting pathologist must be experienced in the assessment of secondary lymphoid tissues of patients with PIDD.

SCORING SYSTEMS

There have been a few attempts to devise validated scoring systems to aid in identifying individuals with PIDD, but both sensitivity and specificity have been poor (Cunningham-Rundles et al., 2004; Hosking & Roberton, 1981; Lyall et al., 1991). Not surprisingly, chronic sinusitis, bronchitis, otitis media, and chronic diarrhea occurred more in immunodeficient patients but were also prevalent in patients with other diseases.

Patient and physician education and support groups have developed awareness-raising tools with respect to PIDD. The "10 Warning Signs" were developed 20 years ago (and recently revised) by the Jeffrey Modell Foundation and the American Red Cross (Table 56.5). These are generally similar to elements of history and physical examination that have emerged as highly associated with PIDD in the analyses mentioned above, as well as in retrospective literature reviews (Wood et al., 2007). These warning signs have not been formally validated, but it is expected that they would have similar (low) sensitivity and specificity as has been found in other analyses using similar criteria (see above). A similar set of "six warning signs for PIDD in adults" has been adapted by the European Society for Immunodeficiency (ESID) to the adult population (www.ESID.org).

A disease-specific scoring system has been established for the hyper-IgE syndrome (see Chapter 38) (Grimbacher et al., 1999). This scoring system has good predictive value for identifying individuals with a high likelihood of having mutations in *STAT3* (Holland et al., 2007). A scoring system has also been developed for distinguishing the various clinical expressions of *WAS* mutation (Ochs & Thrasher, 2006).

Table 56.5 **THE 10 WARNING SIGNS OF PIDDS**

1. Four or more new ear infections within 1 year
2. Two or more serious sinus infections within 1 year
3. Two or more months on antibiotics with little effect
4. Two or more pneumonias within 1 year
5. Failure of an infant to gain weight or grow normally
6. Recurrent, deep skin or organ abscesses
7. Persistent thrush in mouth or fungal infection on skin
8. Need for intravenous antibiotics to clear infections
9. Two or more deep-seated infections, including septicemia
10. A family history of primary immunodeficiency

The presence of two or more warning signs should prompt evaluation for immunodeficiency. From the Jeffrey Modell Foundation (www.jmfworld.com).

FOLLOW-UP EVALUATION

The clinical and laboratory manifestations of many PIDDs are not static. Situations that are unclear initially may evolve into more characteristic constellations of findings that eventually prompt the testing that leads to the definitive diagnosis. Even when a definitive diagnosis is established, there may still be important changes in function over time that require modification of the treatment plan. Furthermore, a wide range of immune-related complications (e.g., autoimmune disease, lymphoproliferative disease, malignancy) may arise during the course of many PIDDs. Some of these result from abnormal regulation and function caused by the fundamental defect, and some from the therapies used to reconstitute function or to treat infections (and other complications). Each of these requires its own set of diagnostic and therapeutic interventions in the context of the underlying PIDD. For all of these reasons, periodic reevaluation of various aspects of immune function is normally undertaken in the course of management of many PIDDs. In addition, follow-up evaluation of PIDD patients should regularly address the course of end-organ damage, including lung, liver, and kidney function, the gastrointestinal tract, the endocrine system, and others.

LABORATORY ASSESSMENT

INNATE IMMUNITY

Phagocytic Cells

PIDDs associated with phagocytic cell defects are discussed in Chapters 50 to 54. The initial evaluation of suspected phagocyte defects must include a blood cell count with differential

and microscopic examination of a peripheral blood smear. Congenital or acquired neutropenias may first be identified in this way. Conversely, a marked leukocytosis may raise suspicion for an adhesion molecule defect. Simple examination of a stained smear may suffice to make a provisional diagnosis of such entities as Chediak-Higashi syndrome or specific granule deficiency, in which morphological abnormalities are characteristic.

Routinely applied clinical tests of phagocyte function include measures of chemotaxis, phagocytosis, and the respiratory burst. One test of chemotaxis uses a device called a Boyden Chamber, in which peripheral blood phagocytic cells are placed in a well above a porous membrane (Chen, 2005). A chemoattractant (e.g., f-Met-Leu-Phe) is placed in the lower chamber and the number of neutrophils passing through the membrane is measured. In another method of measuring chemotaxis, neutrophils are placed in a well in a soft agar medium containing a gradient of a chemotactic factor. The distance migrated by the neutrophils is measured (Mouynet et al., 1994).

Phagocytosis may be determined by a flow-cytometric method in which neutrophils, either purified or in lysed whole blood, are incubated with fluorescent labeled particles such as opsonized yeast and counted in a flow cytometer (Chow et al., 2004). These assays may be performed with or without immunoglobulin and/or complement to assess different mechanisms of phagocytosis. Other assays measure phagocytosis indirectly by stimulation of the respiratory burst (see below).

The most widely applied tests of neutrophil function measure the activity of the NADPH oxidase complex to diagnose the various forms of CGD. The classic example of this is the nitroblue tetrazolium test in which the dye formazan is taken up by neutrophils incubated on endotoxin-coated coverslips (Ochs & Igo, 1973) or loaded into neutrophils via opsonized latex beads (Virella et al., 1990). After activation with phorbol ester or endotoxin, the yellow dye formazan turns blue after reduction in the phagosome. The failure of this color change visualized by microscopic examination or by calorimetric analysis indicates a defect of the oxidase complex (or failure of phagocytosis).

A more quantitative method uses the dye dihydrorhodamine 123 (DHR), which becomes fluorescent upon reduction in the phagosomes of activated neutrophils (Jirapongsananuruk et al., 2003; Vowells et al., 1995). This may be analyzed in a flow cytometer to measure the fluorescence intensity and the proportion of responding cells (Fig. 56.1). Both methods are also useful for carrier detection for X-linked CGD in females who have two populations of neutrophils (with and without oxidase function) due to random X-chromosome inactivation.

In the chemiluminescence assay, phagocytosis and respiratory burst function may be measured simultaneously. Neutrophils are activated with phorbol ester or with opsonized particles. Oxidase activity leads to spontaneous emission of photons, which can be enhanced with addition of a luminophore. The light emitted can be measured in a liquid scintillation spectrometer or other light-sensing instrument (Dahlgren & Karlsson, 1999).

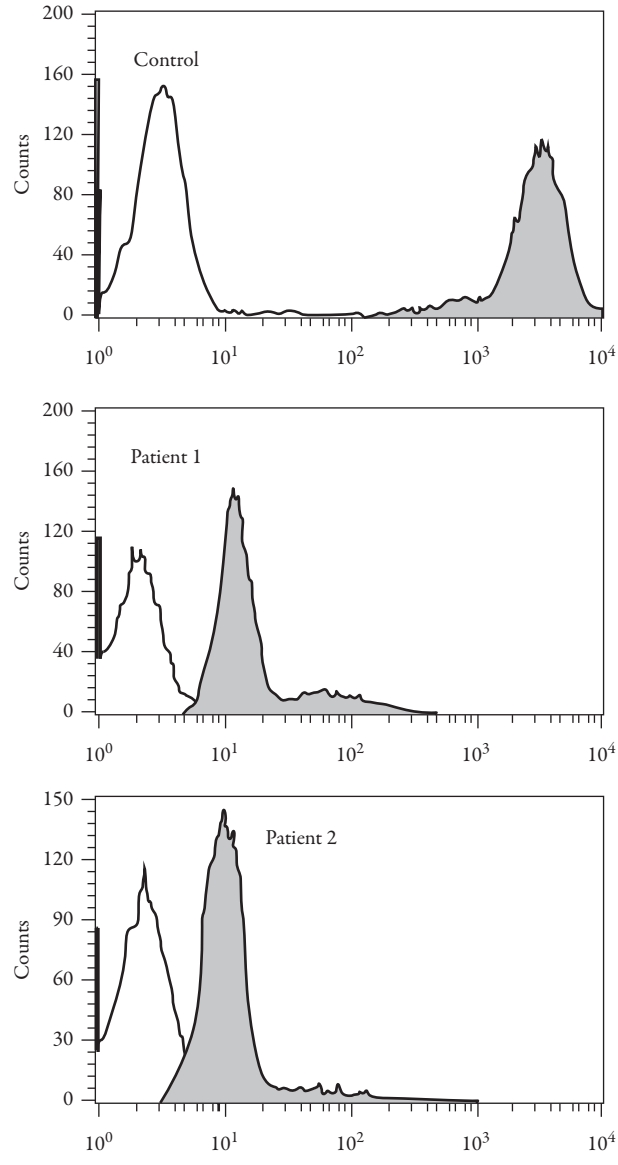

Figure 56.1 DHR assay histograms. The x-axis is fluorescence intensity and the y-axis is the count of cells. The empty peak at the left represents unstimulated cells and the filled peak to the right is after PMA stimulation. The upper panel shows the results for neutrophils from a healthy adult blood donor; the lower two panels show the activity of cells from two sisters with CGD due to defects of p47phox. Clearly, the shift in fluorescence of the patients' cells is markedly less than the control. (Images provided by Petar Zarev, MD, PhD, Laboratory Scientist, Cellular Immunology and Flow Cytometry Laboratory, Department of Laboratory Medicine, Boston, Children's Hospital.)

A bacterial killing assay measures the ability of neutrophils to take up viable bacteria from solution and kill them intracellularly. Bacteria (*Staphylococcus aureus* is often used) are incubated in various conditions (e.g., with or without immunoglobulin or complement) with neutrophils. At various time points, the cells are collected and lysed and the number of viable bacteria is counted by colony formation on agar (Hampton & Winterbourn, 1999). Similar assays may be performed using *Candida albicans*.

Leukocyte adhesion defects (LADs) are rare disorders of neutrophil migration due to mutations of structure or function of surface glycoproteins required for neutrophil tethering and adhering to blood vessel walls and diapedesis into areas of infection (see Chapter 53). LAD type I is caused by a defect of the CD18 integrin β_2 chain; LAD type II results from mutations of a fucosyl transferase enzyme leading to lack of surface expression of sialyl-Lewis^X (SLe^X), a ligand for E-selectin (CD62E). The absence of these molecules on the neutrophil surface may be determined by flow cytometry using appropriate monoclonal antibodies: anti-CD18 for LAD type I and anti-CD15 for Sle^X for LAD type II.

Complement

Disorders of the complement system are discussed in Chapter 55. The complement system may be activated along three distinct sequences of reactions, the so-called classical, alternative, and lectin pathways. All of these converge into the same sequence of terminal reactions after activation of C3. Defects of all pathways have been associated with clinical disease, although the importance of the lectin pathway in this regard remains the subject of some controversy.

The standard assay of classical pathway complement activity is called the CH50 (classical pathway hemolysis 50 percent). In its original form, various dilutions of a serum specimen are added to a suspension of sheep red blood cells (SRBC) sensitized with a complement-fixing immunoglobulin such as rabbit IgM anti-SRBC. The amount of hemolysis is measured in a spectrophotometer and the result is reported as the reciprocal of the calculated dilution that yields lysis of 50 percent of the SRBC (Schur, 1983).

More modern methods may use artificial liposome targets loaded with the enzyme glucose-6-phosphate dehydrogenase and labeled with a defined antigen such as dinitrophenyl (DNP). Liposomes are lysed by the addition of complement-fixing anti-DNP antibody and serum. The released enzyme acts on glucose-6-phosphate and nicotinamide adenine dinucleotide in solution and the color change is measured in a spectrophotometer (Yamamoto et al., 1995)

Analogous to the CH50, the standard assay of the alternative pathway is the AH50. Rabbit red blood cells are susceptible to spontaneous lysis by the alternative pathway in the absence of antibody (Platts-Mills & Ishizaka, 1974). More modern variations have also been developed for measurement of alternative pathway function. For example, serum is placed in a well in a plate covered with agar containing a suspension of guinea pig erythrocytes. As complement components diffuse into the agar, they cause lysis of the red cells. The result is determined by the diameter of the ring of lysis around the well (Arnaout et al., 1977).

Hemolytic methods have also been developed to measure the function of the lectin pathway (Herpers et al., 2009). A different commercially available functional test of the lectin pathway uses a solid-phase method in which plates are coated with mannan and incubated with serum under conditions that permit mannose-binding lectin to be fixed and activated while C1q binding is inhibited. Purified C4 is added and converted to soluble C4a and C4b, which adheres to the plate. The amount of C4b deposited may be measured using a labeled monoclonal antibody to C4b (Petersen et al., 2001).

When a complement deficiency is suspected, both CH50 and AH50 tests may be performed. If the CH50 only is abnormal, this indicates an abnormality of one of the early classical pathway components. If the AH50 only is abnormal, one of the alternative pathway components is deficient. If both CH50 and AH50 are low, then one of the terminal components is deficient (Wen et al., 2004). When these functional tests identify blocks in complement cascades, the levels of individual components may be measured by standard immunoassay methods. In some instances, nonfunctional immunoreactive protein may be present in serum. In these cases, the functional deficit may be demonstrated by measuring the ability of serum to restore function in an assay system in which a single complement component has been depleted by a monoclonal antibody.

NK Cells

NK cells usually represent approximately 5 to 25 percent of peripheral blood lymphocytes. Their function is the lysis of target cells after activation through poorly understood activating mechanisms in the absence of inhibitory signals through the killer inhibitory receptors (KIRs) (Bryceson & Long, 2008). While several PIDDs present with decreased peripheral NK-cell numbers, these disorders are usually dominated by severely disturbed functions of other components of the adaptive immune system (e.g., SCID, CID, subgroups of CVID patients). Isolated defects of NK-cell number and/or function are relatively rare (Orange, 2006). In contrast, disturbed NK-cell function is one of the hallmarks of hemophagocytic syndromes, including perforin deficiency (familial hemophagocytic lymphohistiocytosis type 2), MUNC13b deficiency, Rab27a deficiency (a form of Griscelli syndrome), Chediak-Higashi syndrome due to mutations in *LYST*, and X-linked lymphoproliferative syndrome due to mutations in *SH2D1A* (see Chapters 54 and 44, respectively). The assessment of NK-cell function will be discussed below in the section on cytotoxicity.

Toll-like Receptor Function

The Toll-like receptors (TLRs) are one group of pattern-recognition receptors that initiate innate cellular immune protective mechanisms and participate in and modulate adaptive immune mechanisms as well. TLRs and the PIDDs associated with their dysfunction are discussed in Chapter 36. TLR function may be assessed by measuring cytokine production by peripheral blood mononuclear cells (PBMCs) stimulated with various ligands for the individual TLRs. Figure 56.2 shows an example of defective interferon (IFN)-α production by PBMCs from a patient with interleukin (IL)-1 receptor-associated kinase 4 (IRAK-4) deficiency (McDonald et al., 2006). Commercially available tests of TLR function use similar ligands and may measure production of a variety of

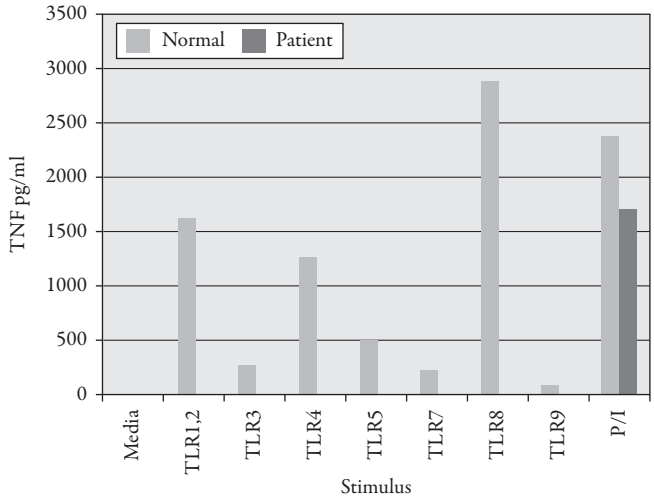

Figure 56.2 Cytokine production after stimulation of TLRs. PBMCs were stimulated with ligands specific for each of the TLRs listed, as well as a nonspecific activating stimulus, PMA and ionomycin (P/I). TNF production of cells from a healthy control was compared to a patient with IRAK4 deficiency. The patient has negligible TNF production with all TLR stimuli. (Reproduced from McDonald et al., 2006, with permission.)

cytokines, such as IFN-β, IL-6, and tumor necrosis factor (TNF) in culture supernatants using standard immunoassays.

Most TLRs signal through the adapter proteins MyD88 and IRAK4 (Barton & Kagan, 2009). Defects of either of these molecules lead to a global impairment of TLR signaling, with the possible exception of TLR3 (Fig. 56.2) (McDonald et al., 2006, von Bernuth et al., 2008). Defects of TLR3 itself and UNC93B lead to selective deficits of TLR3 signaling (Casrouge et al., 2006; Zhang et al., 2007).

ADAPTIVE IMMUNITY

Serological Assays

Serological assays are mainly used to determine total and specific immunoglobulin levels in the serum of patients with suspected immunodeficiency. The general availability and the low cost render the determination of IgG, IgA, and IgM serum levels an excellent screening parameter in patients with suspected humoral immunodeficiency. Immunoglobulins are produced by different subsets of B cells, usually after antigen-specific stimulation. While IgM serum levels are considered to represent a constantly produced, so-called "natural" response, as well as the early antigen-triggered response (Boes, 2000), antigen-specific IgG and IgA are mainly produced by plasma cells derived from class-switched memory B cells after their differentiation in germinal centers within secondary lymphoid tissues. Class-switching to IgG and IgA requires B-cell intrinsic machinery and is largely T-cell–dependent. Thus, patients with either a disturbed class-switch function or absent T-cell help present with severely reduced IgG and IgA, but normal or even elevated IgM (Durandy, 2009) (see Chapters 26 and 27). The assessment of serum immunoglobulins must use age-adjusted normal values, especially in children (Table 56.6). Due to possibly transient dysregulation, when abnormalities are relatively mild, determination of serum Ig levels should be repeated twice with an interval of at least 6 weeks in between, based on the half-life of IgG.

Hypogammaglobulinemia is found in several PIDDs (Table 56.7), of which CVID is the most common form (see Chapter 28). For the diagnosis of CVID, secondary hypogammaglobulinemia due to protein-losing enteropathy and severe proteinuria has to be excluded by clinical presentation and additional studies such as serum albumin levels and urinalysis. Other important elements of the differential diagnosis include lymphoma, especially in late-onset hypogammaglobulinemia and drug-induced hypogammaglobulinemia.

IgG consists of four subclasses (IgG1–4), which differ slightly in their functions (complement activation, Fc receptor binding), half-life, and antigen specificity. Determination of IgG subclasses should be reserved for specific subgroups of patients, including those with the clinical presentation of humoral deficiency with normal or only borderline reduction of IgG. Established clinically relevant PIDDs include combined IgG2/IgG4 deficiency and extreme reductions in IgG1 or IgG2; the relevance of an isolated IgG3 reduction and especially of an IgG4 reduction is less well demonstrated (Olinder-Nielsen et al., 2007).

Reductions or elevations of specific isotypes are important differential biomarkers in PIDDs. IgA-deficient patients (see Chapter 28) with increased susceptibility to infection should be evaluated for additional IgG subclass deficiency (see below). The selective reduction of IgM serum levels (<0.2 g/L) is reported in small cohort studies (Goldstein et al., 2008), but the pathogenesis is not understood. In contrast, the increase of serum IgM levels combined with severely reduced IgG and IgA levels is the hallmark of the immunoglobulin class-switch disorders due to either B-cell intrinsic defects affecting the necessary steps of DNA recombination (Durandy, 2009) or defects in B-cell costimulation by activated T cells as a result of mutations in CD40 or CD40L. B-cell intrinsic defects can be defined by in vitro class-switch assays (see below). CD40 deficiency may be assessed by flow-cytometric evaluation of surface expression on B cells; CD40L deficiency is analyzed on activated CD4 T cells using anti-CD40L monoclonal antibodies or a soluble CD40–Ig construct.

There is no significance of very low levels of IgE. However, elevated levels of IgE are associated with several forms of PIDD (see the section on atopy above and Chapter 38).

There is no established clinical utility of routine measurement of serum IgD levels in the evaluation of PIDDs. Low IgD may be seen in association with low levels of C2 (Calvo et al., 2000), although this has not been demonstrated to contribute to infection susceptibility in this setting or in any other. Elevated levels of IgD (>0.05 g/L) are seen in mevalonate kinase deficiency (hyper-IgD syndrome, Chapter 33) and other periodic fever syndromes (Haas & Hoffmann, 2006).

To evaluate the antibody-forming capacity in patients with the typical clinical presentation of humoral immunodeficiency with only a selective decrease in or normal levels of immunoglobulins, the analysis of specific antibody responses

Table 56.6 AGE-ADJUSTED REFERENCE RANGES FOR SERUM IMMUNOGLOBULINS (G/L)

AGE	IGG	IGA	IGM	
0–1 months	7.00–13.00	0.00–0.10	0.05–0.30	
1–4 months	2.80–7.50	0.06–0.50	0.15–0.70	
4–7 months	2.00–12.00	0.80–0.90	0.10–0.90	
7–13 months	3.00–15.00	0.16–1.00	0.25–1.15	
13 months–3 years	4.00–13.00	0.20–2.30	0.30–1.20	
3–6 years	6.00–15.00	0.50–1.50	0.22–1.00	
6 years to adult	6.39–13.44	0.70–3.12	0.56–3.52	
AGE	IGG1	IGG2	IGG3	IGG4
Cord	4.35–1.084	1.43–4.53	0.27–1.46	0.01–0.47
0–3 months	2.18–4.96	0.40–1.67	0.04–0.23	0.01–1.20
3–6 months	1.43–3.94	0.23–1.47	0.04–1.00	0.01–1.20
6–9 months	1.90–3.88	0.37–0.60	0.12–0.62	0.01–1.20
9 months—2 years	2.86–6.80	0.30–3.27	0.13–0.82	0.01–1.20
2–4 years	3.81–8.84	0.70–4.43	0.17–0.90	0.01–1.20
4–6 years	2.92–8 16	0.83–5.13	0.8–1.11	0.02–1.12
6–8 years	4.22–8.02	1.13–4.80	0.15–1.33	0.01–1.38
8–10 years	4.56–9.38	1.63–5.13	0.26–1.13	0.01–0.95
10–12 years	4.56–9.52	1.47–4.93	0.12–1.79	0.01–1.53
12–14 years	3.47–9.93	1.40–4.40	0.23–1.17	0.1–1.43
Adult	4.22–12.92	1.17–7.47	0.41–1.29	0.10–0.67

These are normal ranges (5th–95th percentiles) from the laboratories of Boston, Children's Hospital, MA. Normal ranges are method-dependent and should be validated for each laboratory. These reference ranges are intended for educational purposes only.

Table 56.7 PIDDS ASSOCIATED WITH HYPOGAMMAGLOBULINEMIA

SCID (X-linked and autosomal recessive forms) including ADA deficiency
Agammaglobulinemia (X-linked and autosomal recessive forms)
Common variable immunodeficiency
Class-switch deficiency disorders (defects of CD40L, CD40, AID, UNG, PMS2, NEMO)
X-linked lymphoproliferative syndrome
Some forms of radiosensitive PIDD (Njimegen breakage syndrome, DNA ligase IV and Cernunnos deficiencies)
Facultative manifestation of other PIDD

is the next step. At initial evaluation, it is generally appropriate only to measure antibodies to antigens to which patients are exposed by vaccination or are prevalent in the population (e.g., EBV, cytomegalovirus). Two different qualities of the humoral response should be tested by assessing antiprotein and antipolysaccharide responses (Bonilla et al., 2005). The former usually include antitetanus or diphtheria toxoid responses, since a high prevalence of vaccination can be assumed in most countries.

In a few centers, responses to neoantigens like bacteriophage X174 (Ochs et al., 1992) or keyhole limpet hemocyanin (KLH) (Kondratenko et al., 1997) are available for testing. Rabies vaccine has also been used as a neoantigen in the assessment of humoral immunodeficiency (van Zelm et al., 2006). Since patients have no prior exposure to these antigens, primary as well as recall responses can be standardized. In addition, the use of neoantigens permits the evaluation of patients receiving IgG replacement therapy, since no preformed antibodies interfere with the response or the diagnostic detection. However, except for a few centers, neither advantage has yet led to a widespread use of neoantigen immunization in the routine clinical assessment of PIDD.

For the assessment of the antipolysaccharide response, the available 23-valent unconjugated (pure polysaccharide) pneumococcal vaccine (Pneumovax®, Pnu-Immune-23®) has been routinely used (Paris & Sorensen, 2007) in children above the age of 2 years and adults. The limitations of this approach are that not many laboratories perform serotype-specific assays, and there is not yet consensus on rigorously defined criteria of normal response (Jeurissen et al., 2007). Sanders et al. defined a normal response to polysaccharides as a twofold or greater increase in serum antibody titer and a postimmunization antibody concentration of at least 20 percent (20 U/L) of a hyperimmune plasma pool of healthy volunteers immunized with Pneumovax® (Sanders et al., 1993). Nonresponsiveness was defined as failure to generate antibody to five or more of the seven serotypes tested. Other authors recommend a fourfold rise in titer as one criterion (Bonilla et al., 2005; Paris & Sorensen, 2007). However, if

the initial level is relatively high, a fourfold rise may not occur, even in normal individuals. Furthermore, children older than 2 years should respond to 50 percent or more types, while all individuals older than 5 years should respond to 70 percent or more types (Bonilla et al., 2005; Paris & Sorensen, 2007). A level of 1.0 to 1.3 μg/mL is considered protective against invasive disease. Methods of measurement vary among laboratories, and it is not clear that these criteria can be applied in all cases.

As an alternative, a polysaccharide vaccine against *Salmonella typhi* (Typhum Vi) has become available, but the established assay (Ferry et al., 2004) is not available in most centers. These assays are used to detect patients with specific polysaccharide antibody deficiency (SPAD) and are indicated in the evaluation of patients with recurrent infection with encapsulated bacteria, usually *Haemophilus influenzae* or pneumococci.

Cellular Assays

After the evaluation of the differential blood count to determine the total lymphocyte number, a basic lymphocyte subset flow-cytometry profile is often applied in patients with suspected PIDD. This represents the essential assay in the differential diagnosis of SCID, separating patients according to the presence or absence of T cells, B cells, and NK cells and directing evaluation toward the respective underlying genetic defects (see Chapter 9). Flow cytometry is also important for the diagnosis of agammaglobulinemia (Chapter 25), which is characterized by extremely low (<1 percent of lymphocytes) or absent B cells.

The primary panel should consist of at least markers identifying $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, $CD19^+$ or $CD20^+$ B cells, and $CD16^+$ or $CD56^+$ NK cells. As is the case with serological assays, age-matched normal values need to be applied (Tables 56.8 and 56.9) (van Gent et al., 2009). A potential pitfall in the diagnosis of SCID is the presence of maternal T cells in the circulation, giving the false impression that host T-cell development is occurring. However, these cells may be identified by the memory T-cell marker CD45RO, which is not normally found on most peripheral blood T cells in newborns (see below). Hypomorphic SCID variants and a host of other PIDDs with abnormal T-cell number and function may have less severely diminished lymphocyte populations.

Some abnormalities of the basic flow-cytometry panel may suggest specific diagnoses in certain clinical contexts. For example, selective reduction in $CD8^+$ T cells should be analyzed for ZAP70 deficiency (see Chapter 14), CD8α deficiency (Chapter 18), or MHC class I expression defects (Chapter 16). In the latter case, $CD8^+$ T cells need to be carefully analyzed for αβTCR expression, since normal CD8 numbers may be detected due to the contribution of an expanded γδ $CD8^+$ T-cell population. HIV-negative T-cell lymphopenia mainly affecting $CD4^+$ T cells may be idiopathic CD4 lymphopenia (Zonios et al., 2008). In rare cases, a defect in the expression of the src family kinase lck has been described (Goldman et al., 1998). In MHC class II deficiency, $CD4^+$ T-cell counts are only mildly reduced.

More detailed T-cell phenotyping may include the separation of $CD4^+$ T cells into $CD45RA^+$ naïve and $CD45RO^+$ memory or activated T cells (Tables 56.8 and 56.9 and Fig. 56.3). This separation is especially helpful in the context of identifying engrafted maternal T cells in SCID patients, since these cells are almost exclusively $CD45RO^+$, while $CD4^+$ T cells of infants are usually 60 to 95 percent $CD45RA^+$ (naïve) T cells (van Gent et al., 2009). The small number of endogenous $CD4^+$ T cells of (S)CID patients often exhibit an activated phenotype expressing CD45RO and HLA-DR. In CVID, the absolute reduction of naïve $CD4^+CD45RA^+$ T cells has also been suggested as a means of classifying patients. Those with reduced $CD4^+CD45RA^+$ T-cell counts often present with a more complicated clinical course due to inflammatory and lymphoproliferative manifestations (Giovannetti et al., 2007).

Naïve T cells that have recently emigrated from the thymus express CD31 (platelet endothelial cell adhesion molecule 1 [PECAM-1]) (Kohler & Thiel, 2009). $CD4^+$ cells that express CD45RA and CD62L (L-selectin) are also enriched for recent thymic emigrants. The enumeration of these cells may be helpful in determining the level of thymic function in patients with DiGeorge syndrome or SCID with maternal T-cell engraftment, Omenn syndrome, or other "leaky" phenotypes with oligoclonal T-cell populations (Markert et al., 2004; Poulin et al., 1999).

Molecular methods to identify recent thymic emigrant T cells have also been developed. During the process of T-cell development in the thymus, T-cell receptor (TCR) gene segments rearrange to assemble mature TCR genes. The DNA between these segments is excised and ligated into a closed loop that remains as an episomal DNA fragment that is preserved within the nucleus. This circular fragment is known as a T-cell-receptor excision circle (TREC) (Dion et al., 2007). TRECs do not replicate as cells divide, so the content of TRECs within naïve cells that have just exited the thymus is much higher than in cells that have expanded in the periphery. TRECs can be expanded by PCR methods and serve as another measure of thymic function. Normal values have been established (Gent et al. 2009). The PCR methods for detecting TRECs can be also applied to the dried blood spots that are collected as part of newborn screening programs for genetic/metabolic diseases. Pilot programs of screening for SCID by the detection of TRECs in newborn dried blood spots have been initiated in several states in the United States (Baker et al., 2009) and have shown promising results (Verbsky et al., 2012). The level of TRECs in SCID infants is extremely low or absent due to the rudimentary nature of the thymus in the majority.

Additional analyses of T-cell subpopulations helpful in the differential diagnosis of PIDD include the increase in double-negative ($CD4^-CD8^-$) $\alpha\beta TCR^+$ T cells (DN T cells, Fig. 56.3) in ALPS patients (Chapter 30), the reduction of NK T cells ($CD3^+CD56^+$) in XLP (Chapter 44) (Pasquier et al., 2005; Rigaud et al., 2006), $CD4^+CD25^+FOXP3^+$ (or alternatively $CD4^+CD25^+CD127^-$) regulatory T cells in IPEX patients (Chapter 32) (Bennett et al., 2001), and reduction of TH17 T cells (producing IL-17) in STAT3-deficient hyper-IgE syndrome (see Chapter 38) (Ma et al., 2008). None of these changes is specific, and they need to be interpreted

Table 56. 8 REFERENCE VALUES OF LYMPHOCYTE SUBPOPULATIONS IN PERIPHERAL BLOOD OF HEALTHY PEDIATRIC CONTROLS

A. PERCENTAGES OF TOTAL T CELLS AND T-CELL SUBSETS

	NO.	CORD BLOOD	0–6 MONTHS	6–12 MONTHS	1–2 YEARS	2–3 YEARS	3–4 YEARS	4–6 YEARS	6–9 YEARS	9–12 YEARS	12–15 YEARS	15–18 YEARS
Lymphocytes (of leukocytes)	98	ND	63.1 (50.1–70.1)	58.2 (48.4–76.5)	63.4 (43.0–65.2)	47.7 (33.4–62.8)	52.0 (36.3–60.5)	39.9 (30.2–59.2)	46.3 (26.0–49.6)	42.0 (34.5–48.2)	40.8 (24.2–51.3)	34.3 (27.0–44.1)
Total $CD4^+$ T cells	109	46.7 (40.2–61.9)	43.2 (37.6–46.4)	43.5 (29.8–63.4)	42.4 (33.0–55.0)	34.5 (28.1–43.2)	37.1 (18.3–42.7)	38.2 (26.8–42.3)	31.5 (26.5–41.4)	36.2 (28.4–44.4)	35.0 (30.4–52.9)	38.2 (29.3–52.9)
Regulatory T cells ($CD127^-$-$CD25^+CD4^+$)	81	5.2 (3.5–7.0)	6.8 (4.9–9.8)	5.5 (3.6–9.0)	5.1 (3.7–6.7)	5.0 (2.9–7.4)	4.9 (3.3–6.8)	4.9 (3.1–6.1)	5.4 (2.3–7.7)	4.0 (2.6–6.1)	4.7 (2.8–7.2)	3.1 (2.2–4.1)
Naïve $CD4^+$ T cells ($CD27^+CD45RO^-$ $CD4^+$)	109	94.5 (91.9–98.1)	92.6 (89.6–94.6)	89.7 (83.5–94.7)	87.4 (82.3–95.1)	78.8 (71.5–84.2)	74.8 (64.5–83.8)	72.7 (57.8–81.0)	65.1 (55.6–75.8)	62.7 (53.4–74.7)	60.2 (49.3–72.0)	62.0 (49.4–71.9)
Recent thymic emigrants ($CD31^+$)[a]	81	78.1 (71.7–81.3)	71.3 (60.5–81.8)	71.5 (58.8–83.0)	66.0 (48.4–76.3)	69.3 (65.0–79.5)	79.1 (65.2–83.4)	79.3 (71.6–85.3)	76 (61.0–84.2)	67.1 (57.9–80.5)	69.8 (50.7–78.9)	63.9 (51.4–79.1)
Total memory $CD4^+$ ($CD45RO^+$) T cells	109	5.1 (1.9–8.1)	7.0 (5.3–10.1)	10.3 (5.1–15.2)	12.3 (4.6–17.3)	21.0 (15.6–27.5)	24.5 (15.6–35.4)	26.9 (18.9–42.1)	34.3 (24.0–43.4)	33.9 (24.5–46.4)	39.5 (27.9–48.8)	37.5 (27.3–49.8)
Total $CD8^+$ T cells	109	16.3 (14.3–21.3)	14.9 (7.7–22.0)	14.2 (11.0–17.8)	15.5 (14.0–26.0)	16.7 (12.2–22.6)	21.3 (14.7–27.2)	24.5 (17.5–31.1)	20.4 (13.8–28.8)	22.4 (16.4–36.2)	18.7 (13.6–23.5)	18.8 (13.5–22.0)
Naïve $CD8^+$ T cells ($CD27^+CD45RO^-$)	109	95.4 (90.4–98.1)	94.9 (78.5–97.1)	94.4 (68.7–96.6)	95.7 (77.5–98.5)	86.7 (57.1–91.4)	74.7 (54.6–90.6)	64.6 (53.2–87.2)	75.8 (57.0–83.7)	74.0 (49.2–82.7)	74.7 (62.3–86.3)	64.3 (48.6–87.5)
Central memory $CD8^+$ T cells ($CD27^+CD45RO^+$)	109	4.2 (1.7–8.8)	3.7 (2.6–14.6)	5.2 (3.1–11.6)	4.1 (1.0–8.3)	10.5 (5.9–22.3)	14.2 (5.5–23.3)	16.6 (8.5–38.9)	16.6 (9.2–22.6)	18.3 (11.5–30.7)	19.1 (12.2–27.2)	24.2 (9.8–37.6)
Effector memory $CD8^+$ T cells ($CD27^-$-$CD45RO^+CD8^+$)	109	0.1 (0.0–0.1)	0.1 (0.0–3.1)	0.1 (0.1–7.1)	0.1 (0.0–2.8)	0.8 (0.3–6.8)	1.3 (0.4–12.7)	4.3 (0.4–6.0)	2.3 (0.7–14.0)	1.6 (0.7–7.5)	1.2 (0.8–6.2)	3.7 (0.2–6.9)
Effector $CD8^+$ T cells ($CD27^-CD45RO^-$ $CD8^+$)	109	0.7 (0.0–1.1)	0.3 (0.1–6.7)	0.3 (0.2–12.7)	0.6 (0.1–11.6)	2.5 (0.6–16.4)	2.8 (0.8–21.7)	4.3 (0.6–28.9)	3.8 (0.9–17.9)	6.1 (1.5–20.8)	1.4 (0.8–13.2)	6.6 (0.8–14.0)

Values are presented as medians (5th–95th percentiles). Total CD4 and CD8 T cells are percentage of lymphocytes; subsets are percentage of total CD4 or CD8 T cells. No. = number of individuals; ND = not determined. (Adapted from van Gent et al., 2009, with permission.)

[a]Expressed as a proportion of naïve $CD4^+$ cells.

B. COUNTS OF TOTAL T CELLS AND T-CELL SUBSETS

	NO.	CORD BLOOD	0–6 MONTHS	6–12 MONTHS	1–2 YEARS	2–3 YEARS	3–4 YEARS	4–6 YEARS	6–9 YEARS	9–12 YEARS	12–15 YEARS	15–18 YEARS
Leukocytes ($\times 10^9$/L)	98	ND	10.8 (4.7–14.8)	12.1 (6.6–13.6)	8.7 (7.4–14.3)	9.5 (6.6–12.9)	8.5 (5.0–11.2)	8.4 (6.7–11.9)	7.0 (4.0–12.5)	6.0 (5.3–11.2)	5.9 (4.1–8.3)	5.9 (4.8–7.4)
Lymphocytes ($\times 10^9$/L)	98	ND	5.2 (3.2–9.8)	6.4 (3.8–9.3)	5.6 (4.6–6.0)	4.4 (2.8–6.4)	3.7 (2.2–5.9)	3.4 (2.8–4.8)	3.0 (1.8–5.0)	2.7 (2.1–4.0)	2.4 (1.5–2.8)	2.1 (1.5–2.7)
Total $CD4^+$ T cells	98	ND	1,985 (1,294–4,012)	2,721 (1,327–4,455)	2,073 (1,902–2,977)	1,469 (925–2,477)	1,044 (646–2,331)	1,186 (1,000–1,931)	992 (641–1,453)	970 (695–1,473)	904 (608–1,217)	809 (560–1,067)
Regulatory T cells ($CD127^-CD25^+CD4^+$)	73	ND	208 (64–282)	136 (74–280)	107 (79–162)	63 (42–69)	78 (30–126)	58 (41–86)	47 (18–86)	36 (23–89)	41 (25–64)	26 (19–41)
Naïve $CD4^+$ T cells	98	ND	1,856 (1,164–3,712)	2,512 (1,134–4,204)	1,797 (1,565–2,794)	1,153 (685–2,055)	882 (430–1,871)	894 (630–1,414)	661 (375–1,096)	596 (441–1,109)	523 (311–781)	469 (335–725)
Recent thymic emigrants ($CD31^{+)}$	73	ND	1,684 (781–2,695)	1,920 (788–2,564)	1,188 (961–1,597)	824 (438–1,037)	908 (296–1,446)	644 (493–1,054)	460 (244–894)	442 (313–785)	304 (239–477)	325 (207–585)
Total memory $CD4^+$ ($CD45RO^+$) T cells	98	ND	139 (111–315)	240 (126–378)	245 (121–329)	311 (224–456)	251 (167–499)	399 (234–534)	304 (216–497)	322 (246–564)	357 (252–417)	273 (216–490)
Total $CD8^+$ T cells	98	ND	747 (394–1,865)	850 (593–1,517)	892 (667–1,473)	733 (394–1,197)	868 (365–1,255)	852 (602–1,203)	628 (249–1,440)	652 (426–991)	447 (228–577)	399 (216–499)
Naïve $CD8^+$ T cells ($CD27^+CD45RO^-$)	98	ND	669 (345–1,635)	789 (499–1,423)	834 (637–1,113)	526 (336–927)	673 (248–999)	573 (442–733)	426 (203–961)	448 (267–683)	325 (165–424)	257 (118–312)
Central memory $CD8^+$ T cells ($CD27^+CD45RO^+$)	98	ND	28 (14–260)	40 (26–127)	34 (8–127)	66 (38–239)	105 (32–228)	156 (62–402)	103 (35–256)	100 (72–262)	82 (32–153)	96 (30–166)
Effector memory $CD8^+$ T cells ($CD27^-$-$CD45RO^+CD8^+$)	98	ND	1 (0–18)	1 (0–67)	1 (0–47)	5 (2–77)	18 (2–106)	36 (3–67)	16 (2–109)	18 (3–51)	5 (3–26)	10 (1–26)
Effector $CD8^+$ T cells ($CD27^-CD45RO^-CD8^+$)	98	ND	5 (0–50)	3 (1–123)	5 (1–193)	15 (5–174)	25 (7–218)	40 (5–310)	20 (3–198)	50 (8–155)	5 (3–59)	20 (3–65)

Absolute counts $\times 10^6$ per liter blood, unless stated differently. Values are presented as medians (5th–95th percentiles). No. = number of individuals; ND = not determined. (Adapted from van Gent et al., 2009, with permission).

C. PERCENTAGES OF TOTAL B CELLS AND B-CELL SUBSETS

	NO.	CORD BLOOD	0–6 MONTHS	6–12 MONTHS	1–2 YEARS	2–3 YEARS	3–4 YEARS	4–6 YEARS	6–9 YEARS	9–12 YEARS	12–15 YEARS	15–18 YEARS
Total B cells	90	858 (559–1,054)	1,623 (961–3,679)	1,717 (571–3,680)	1,115 (871–1,553)	1,157 (686–1,732)	709 (359–1,552)	593 (278–1,022)	418 (296–784)	338 (116–555)	284 (119–578)	210 (114–436)
$CD27^{-}IgM^{+}IgD^{+}$ naïve B cells	73	698 (439–800)	988 (627–2,136)	1,378 (420–2,181)	738 (586–955)	757 (346–1,356)	470 (244–724)	344 (149–618)	263 (154–413)	233 (60–300)	193 (83–398)	153 (72–257)
$CD27^{+}IgM^{+}IgD^{+}$ memory B cells	90	25 (24–34)	61 (49–179)	71 (30–396)	116 (40–177)	87 (50–148)	62 (27–210)	62 (32–164)	48 (24–135)	35 (9–109)	21 (10–74)	20 (10–39)
$CD27^{+}IgM^{-}IgD^{-}$ memory B cells	90	0 (0–2)	10 (6–19)	16 (2–42)	21 (16–34)	33 (19–53)	27 (6–113)	24 (7–98)	32 (7–65)	9 (3–34)	7 (3–39)	10 (2–40)

Total B cells as percentage of lymphocytes. B-cell subsets as percentage of total B cells. Values are presented as medians (5th–95th percentiles). No. = number of individuals. (Adapted from van Gent et al., 2009, with permission.)

D. COUNTS OF TOTAL B CELLS AND B-CELL SUBSETS

	NO.	CORD BLOOD	0–6 MONTHS	6–12 MONTHS	1–2 YEARS	2–3 YEARS	3–4 YEARS	4–6 YEARS	6–9 YEARS	9–12 YEARS	12–15 YEARS	15–18 YEARS
Total B cells	90	858 (559–1,054)	1,623 (961–3,679)	1,717 (571–3,680)	1,115 (871–1,553)	1,157 (686–1,732)	709 (359–1,552)	593 (278–1,022)	418 (296–784)	338 (116–555)	284 (119–578)	210 (114–436)
$CD27^{-}IgM^{+}IgD^{+}$ naïve B cells	73	698 (439–800)	988 (627–2,136)	1378 (420–2,181)	738 (586–955)	757 (346–1,356)	470 (244–724)	344 (149–618)	263 (154–413)	233 (60–300)	193 (83–398)	153 (72–257)
$CD27^{+}IgM^{+}IgD^{+}$ memory B cells	90	25 (24–34)	61 (49–179)	71 (30–396)	116 (40–177)	87 (50–148)	62 (27–210)	62 (32–164)	48 (24–135)	35 (9–109)	21 (10–74)	20 (10–39)
$CD27^{+}IgM^{-}IgD^{-}$ memory B cells	90	0 (0–2)	10 (6–19)	16 (2–42)	21 (16–34)	33 (19–53)	27 (6–113)	24 (7–98)	32 (7–65)	9 (3–34)	7 (3–39)	10 (2–40)

Absolute counts × 10^6 per liter blood. Values are presented as medians (5th–95th percentiles). No. = number of individuals. (Adapted from van Gent et al., 2009, with permission.)

Table 56.9 REFERENCE VALUES OF LYMPHOCYTE SUBPOPULATIONS IN PERIPHERAL BLOOD OF HEALTHY ADULT CONTROLS

T-CELL POPULATION		REFERENCE RANGE
$CD3^+$ of lymphocytes	$CD3^+$ T cells	55–83%
$CD4^+$ of lymphocytes	$CD4^+$ T cells	28–57%
$CD8^+$ of lymphocytes	$CD8^+$ T cells	10–39%
$CD4^-CD8^-$ of $CD3^+$alpha/beta TCR^+ T cells	Double-negative T cells (DNT)	0.4–2.2%
$CD4^+CD45RO^+$ of $CD4^+$ T cells	$CD4^+$ memory T cells	29–63%
$CD4^+CD45RA^+$ of $CD4^+$ T cells	$CD4^+$ naïve T cells	32–73%
$CD4^+CD45RA^+CD31^+$ of $CD4^+CD45RA^+$ T cells	Recent thymic emigrants T cells	41–79%
$CD8^+CD27^+CD28^+$ of $CD8^+$ T cells	$CD8^+$ naïve T cells	43–91%
$CD8^+CD27^+CD28^-$ of $CD8^+$ T cells	Early $CD8^+$ effector/memory T cells	3–18%
$CD8^+CD27^-CD28^-$ of $CD8^+$ T cells	Late $CD8^+$ effector/memory T cells	1–41%

B-CELL POPULATION		REFERENCE RANGE
$CD19^+$ in $CD45^+$ lymphocytes	B cells	4.9–18.4%
IgD^+ $CD27^-$ in $CD19^+$ B cells	Naïve B cells	42.6–82.3%
IgD^+ $CD27^+$ in $CD19^+$ B cells	IgM-memory B cells	7.4–32.5%
IgD^- $CD27^+$ in $CD19^+$ B cells	Class switched memory B cells and plasmablasts	6.5–29.1%
IgA^+ in $CD19^+$ B cells	IgA^+ B cells	2.7–13.8%
IgG^+ in $CD19^+$ B cells	IgG^+ B cells	3.6–13.4%
Kappa/lambda ratio	Light chain restriction	1.2–2.0
$CD21^{low}$ $CD38^-$ in $CD19^+$ B cells	$CD21^{low}$ subpopulation	0.9–7.6%
M^{++} D^{++} $CD24^{++}$ $CD38^{++}$ in $CD19^+$ B cells	Transitional B cells	0.6–3.4%
$CD24^-$ $CD38^{++}$ $CD27^+$ in $CD19^+$ B cells	Plasmablasts	0.4–3.6%

Fifth–95th percentiles. T cells: University Medical Centre Freiburg, Clinical Immunology Laboratory, unpublished data. B cells: based on 54 healthy donors (age range 19–61 years) (Wehr et al., 2008). Normal ranges are method-dependent and should be validated for each laboratory. These reference ranges are intended for educational purposes only.

in the context of clinical and other laboratory findings and, finally, confirmed by genetic diagnosis.

Detailed B-cell phenotyping has developed with the discovery of CD27 as a marker for memory B-cell formation (Klein et al., 1998). Agematsu et al. (1998) were the first to describe the reduction of $CD27^+$ B cells in X-linked hyper-IgM syndrome, and Warnatz et al. (2002) were the first to apply B-cell phenotyping in patients with CVID. B-cell phenotyping is now mainly applied in the classification of CVID (see Chapter 28) (Piqueras et al., 2003; Wehr et al., 2008). The current B-cell phenotyping uses mAbs to CD27, IgD, and IgM for the separation of naïve, IgM memory, and switched memory B cells, and mAbs to CD38, CD21, and IgM for the identification of transitional B cells, $CD21^{low}$ B cells, and plasmablasts (Tables 56.8 and 56.9 and Fig. 56.4) (van Gent et al., 2009). Low numbers of switched memory B cells in CVID are associated with autoimmunity, granulomatous disease, and lymphoproliferation. Low levels of switched memory B cells also occur in other immunodeficiencies such as WAS, XLP, STAT3-hyper-IgE syndrome, and so forth (Chaganti et al., 2008; Meyer-Bahlburg et al., 2012; Park et al., 2005). Reduction of IgM memory B cells has been associated with (functional) asplenia and a poor antipolysaccharide response, while the expansion of $CD21^{low}$ B cells is associated with a higher risk of inflammatory and lymphoproliferative manifestations (Rakhmanov et al., 2009). Expansion of transitional B cells is the hallmark of the rare B-cell–activating factor receptor (BAFFR) deficiency (Warnatz et al., 2009) and is frequently found in patients with X-linked lymphoproliferative syndrome (Cuss et al., 2006) as well as idiopathic CD4 lymphocytopenia (Malaspina et al., 2007).

Although not frequently applied in practice, analysis of the differentiation pattern of B-cell precursors in the bone marrow is valuable for the differential diagnosis of patients with severe peripheral B-cell reduction found in variants of SCID or agammaglobulinemia (Noordzij et al., 2002a, 2002b). Staining for immunoglobulin κ or λ light chain expression may be used as a screening parameter for prominent monoclonal B-cell populations.

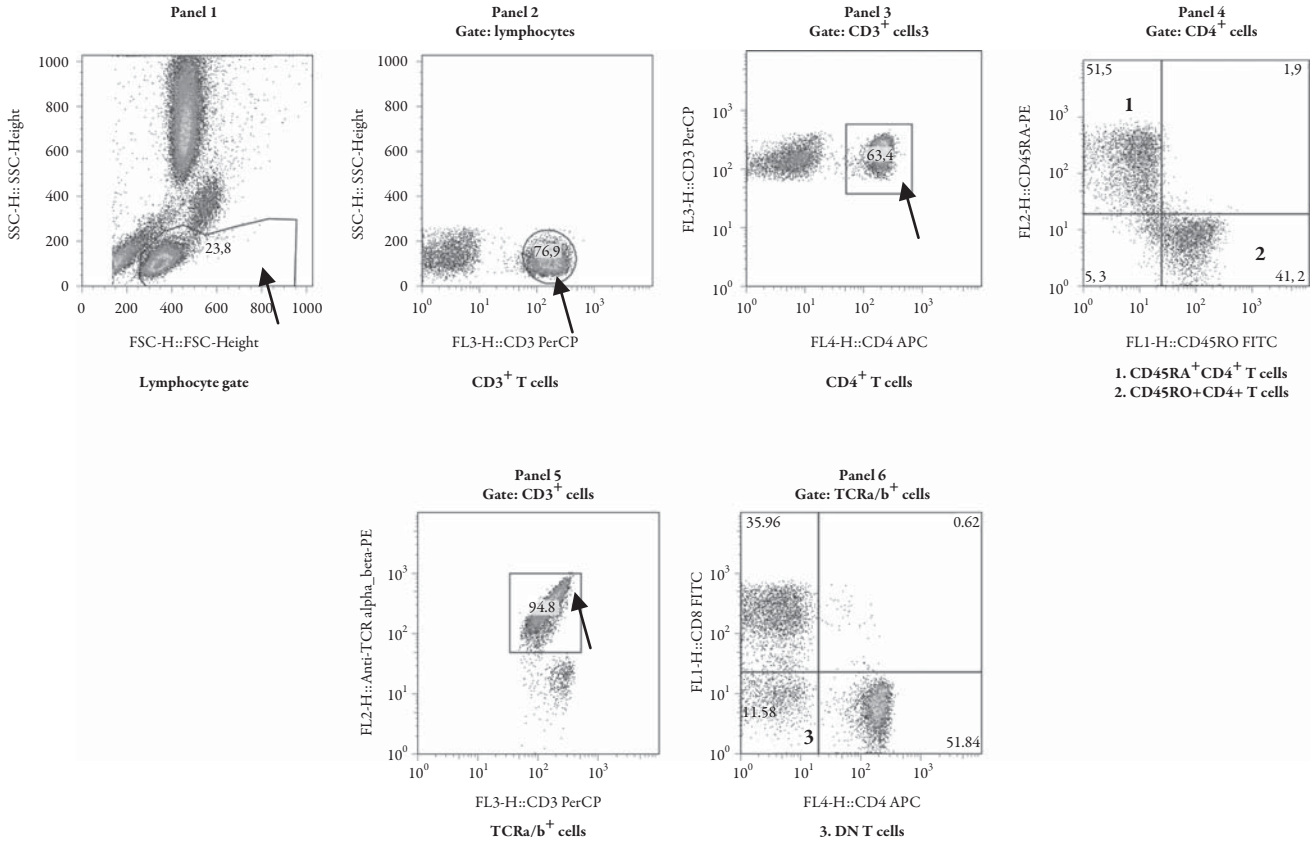

Figure 56.3 T-cell phenotyping. For flow-cytometric evaluation of T cells, lymphocytes gated by forward and side scatter (panel 1) are analyzed for CD3 expression (panel 2). $CD3^+$ T cells are then divided into $CD4^+$ (panel 3) and $CD8^+$ T cells (not shown). CD4 T cells can be further separated into $CD45RA^+$ naïve CD4 T cells (1) and $CD45RO^+$ memory CD4 T Cells (2) (panel 4). Alternatively, by gating on $TCR\alpha/\beta^+$ $CD3^+$ T cells (panel 5), $CD4^-CD8^-$ DN T cells can be determined (3) (panel 6).

FUNCTIONAL TESTS

B Cells: Immunoglobulin Production In Vitro

Human B cells cultured with lectins such as pokeweed mitogen or *S. aureus* Cowan strain will develop into plasma cells producing antibody in vitro. Although not widely applied in clinical practice, it can be a useful method to study the intrinsic capacity of B cells to undergo isotype class-switching and plasma cell differentiation (Livaditi et al., 2007). Other stimuli may be used to study class-switching in vitro. For example, stimulation with cross-linking antibody to CD40 in the presence of IL-4 will lead to production of IgE (Jabara et al., 1995), while stimulation in the presence of transforming growth factor-β leads to production of IgA (Briere et al., 1995).

T Cells

Delayed Hypersensitivity Skin Test

The classic example of this is the Mantoux test for tuberculosis performed by an intradermal injection of the purified protein derivative (PPD) of tuberculin. In individuals infected with *Mycobacterium tuberculosis,* antigen is taken up and presented by skin dendritic cells and antigen-specific T cells are activated and recruit inflammatory cells, leading to cellular infiltration with resulting induration and erythema. The reaction usually develops over 48 to 72 hours. For the purpose of assessing T-cell function diagnostically, the test may be performed with any antigen to which the patient has been exposed (Yates & deShazo, 2001). Examples include tetanus and diphtheria toxoids, streptococcus, *Candida*, *Trichophyton*, and *Proteus*. An area of induration of at least 5 mm is considered positive; in children, some accept 2 mm as a criterion of a positive test (Franz et al., 1976). Infants are able to respond, but the test is not considered generally reliable under age 1 year. Glucocorticosteroids and other immunosuppressive drugs may blunt the response; the reaction may also be suppressed during acute infectious illnesses. In these situations, or when the interpretation of a negative result is unclear, an in vitro method of measuring T-cell function should be used.

T-Cell Proliferation Tests

Peripheral blood T cells may be induced to undergo cell division (proliferate) in vitro by a variety of stimuli. These include nonspecific mitogenic agents such as plant lectins (phytohemagglutinin, concanavalin A, pokeweed mitogen) and antibodies against the CD3 complex, as well as antigens such as tetanus and diphtheria toxoids (Bonilla, 2008; Nisbet-Brown et al., 1987; Stone et al., 2009). Also, phorbol esters and calcium ionophores together induce T-cell activation and

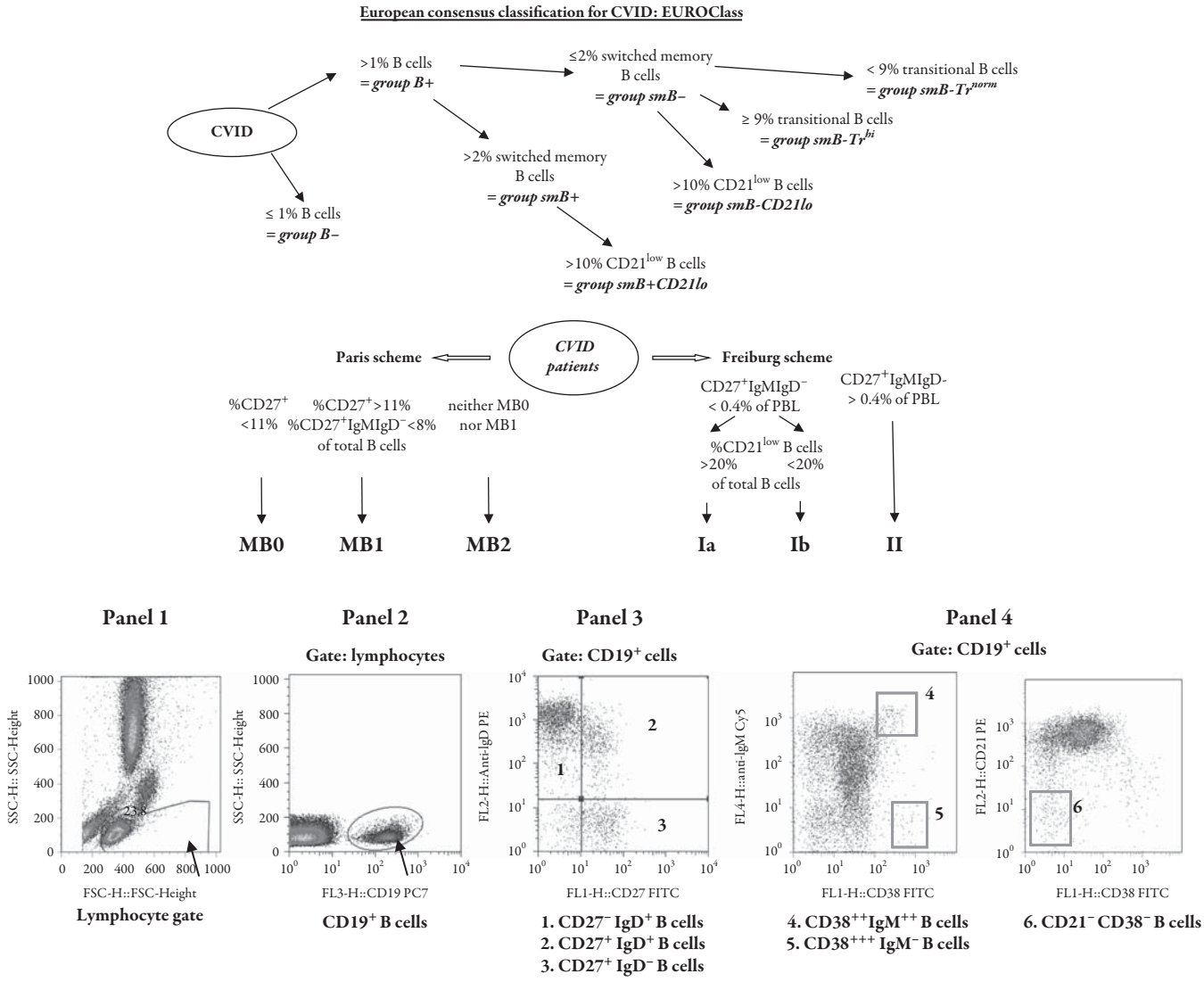

Figure 56.4 B-cell phenotyping. For flow-cytometric evaluation of B cells, lymphocytes gated by forward/side scatter (panel 1) are analyzed for CD19 expression (panel 2). $CD19^+$ B cells are then divided into $CD27^-IgM^+IgD^+$ naïve B cells (1), $CD27^+IgM^+IgD^+$ IgM memory B cells (2), and $CD27^+IgM^-IgD^-$ class-switched memory B cells (3) (panel 3). Additionally, $CD19^+$ B cells can be separated into $CD38^{++}IgM^{++}$ transitional B cells, $CD38^{+++}IgM^-$ plasmablasts, and $CD38^-CD21^{low}$ B cells (6) (panel 4). Classification schemes for CVID based on B-cell phenotyping. Freiburg classification (Warnatz et al., 2002), Paris classification (Piqueras et al., 2003), and EUROClass consensus classification (Wehr et al., 2008).

proliferation bypassing mechanisms of T-cell–receptor signaling (Weiss & Imboden, 1987). Irradiated allogeneic lymphocytes may also be used as a stimulus for proliferation (mixed lymphocyte culture) (Virella et al., 1993). Density gradient-purified PBMCs are incubated with one of these stimuli, and their activity is compared to healthy controls. Nonspecific mitogens will rapidly stimulate the majority of T cells (80 to 100 percent), and these assays can be performed in 3 days. Allogeneic cells induce response in approximately 10 percent of T cells. Specific antigens require processing and presentation and the recruitment of specific T cells that represent only about 1/1,000 T cells or fewer. These latter tests require about 5 to 7 days in culture. The most widely applied method of measuring proliferation uses tritiated thymidine added to the culture. Dividing cells incorporate the radioactive nucleotide into their DNA, and this can be measured in a radiation counter as a surrogate for proliferation.

The ability of T cells to divide in response to mitogens or antigens is affected to varying degrees by discrete lesions in pathways of cellular signaling during activation. This type of test is easily interpreted in an "all-or-none" manner, for example in the diagnosis of SCID. Part of the clinical definition of SCID is an in vitro mitogenic response that is less than 5 to 10 percent of normal (Fig. 56.5) (Railey et al., 2009). The predictive value of proliferation testing for less-severe defects of T-cell function or for the risk of infection is otherwise relatively poor; these tests lack both sensitivity and specificity as a screening tool in diverse patient populations (Stone et al., 2009). This is most likely due to the fact that many clinically important defects of T-cell interaction with other cell types lead to only partial impairment of their ability to divide in culture in response to these stimuli. Similarly, partial impairment of cell division does not necessarily indicate that the cells are incapable of generating effector responses that are sufficient for

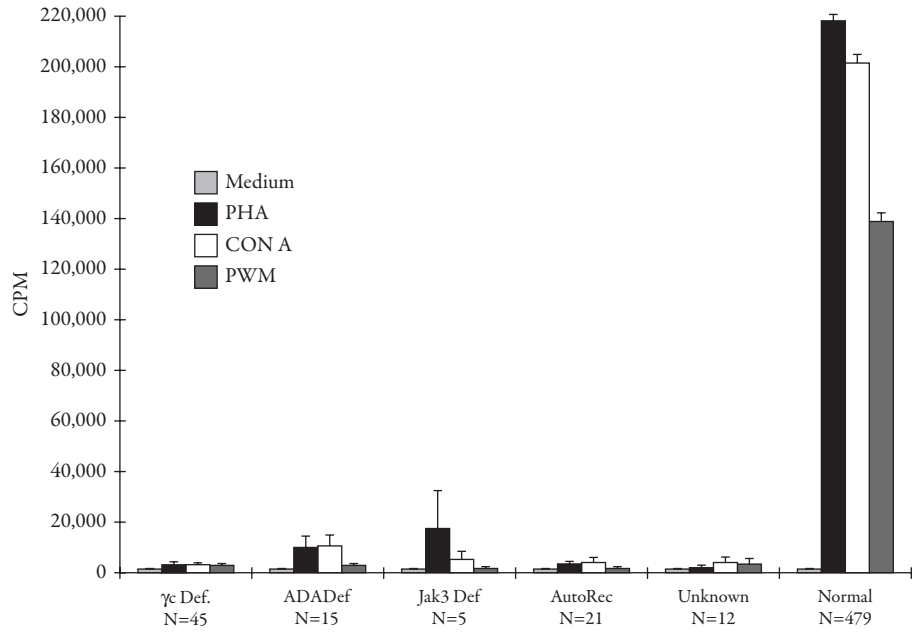

Figure 56.5 Lymphocyte proliferation testing by thymidine incorporation. PBMCs from infants with SCID and healthy adult controls were incubated with PHA, CONA, or PMA, and cell division was measured by incorporation of tritiated thymidine. The CPM of radioactivity incorporated into cells is shown on the y-axis. PBMCs from the infants with the forms of SCID listed failed to divide in response to the mitogens. (Reproduced from Sarzotti-Kelsoe et al., 2009, with permission.)

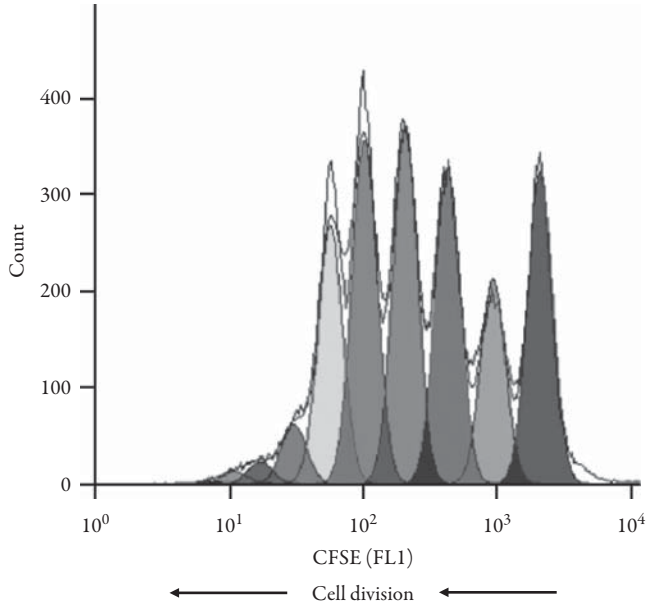

Figure 56.6 Lymphocyte proliferation by CFSE dilution. PBMCs from healthy adults were incubated with CONA. In this flow-cytometry histogram, the peak at the far right represents cells with the highest fluorescence intensity that have the highest internal concentration of CFSE because they have not divided. Each successive peak moving toward the left represents cells that have divided, diluting the CFSE by approximately half with each cycle. There are eight peaks in addition to the peak of undivided cells; thus, this culture contains cells that have undergone up to eight rounds of division. (Reproduced from Wallace & Muirhead, 2007, with permission.)

protection against many pathogens. All proliferative responses must be interpreted in the context of the $CD4^+$ and $CD8^+$ T-cell counts, since severely reduced proportions of $CD4^+$ T cells or preactivated or high numbers of $CD8^+$ T cells reduce the proliferative response in vitro without revealing the proliferative capacity on the cellular level.

A newer method of detecting cell division uses flow cytometry to measure the dilution of a fluorescent tracking dye. One that has been widely used is carboxyfluorescein diacetate succinimidyl ester (CFSE or CFDA-SE) (Lyons et al., 2001). This molecule will diffuse passively into cells where cytoplasmic esterases cleave the acetate moieties, making the molecule fluorescent and lipophobic so it cannot diffuse out. The amount of dye in each cell is reduced as cells divide in culture (Fig. 56.6) (Wallace & Muirhead, 2007). Several other fluorescent dyes with similar properties have also been used. This method may have some advantages over the traditional proliferation assay. With the use of reagents labeled with distinct fluorescent dyes, a multiparameter analysis may be used to track populations of responding cells, such as B cells, or $CD4^+$ or $CD8^+$ T-cell subsets, and so forth. Reagents may also be targeted to cytokines or surface molecules to provide additional functional readouts in addition to proliferation (Fig. 56.7) (Munier et al., 2009). These methods are not yet widely applied in clinical settings.

Although there is generally good correlation between proliferation assays using tritiated thymidine and methods using fluorescent dyes, the dye molecules may inhibit some processes of cellular activation and may cause cells with low proliferation in a tritiated thymidine assay to cease to divide altogether (Last'ovicka et al., 2009). The performance of this

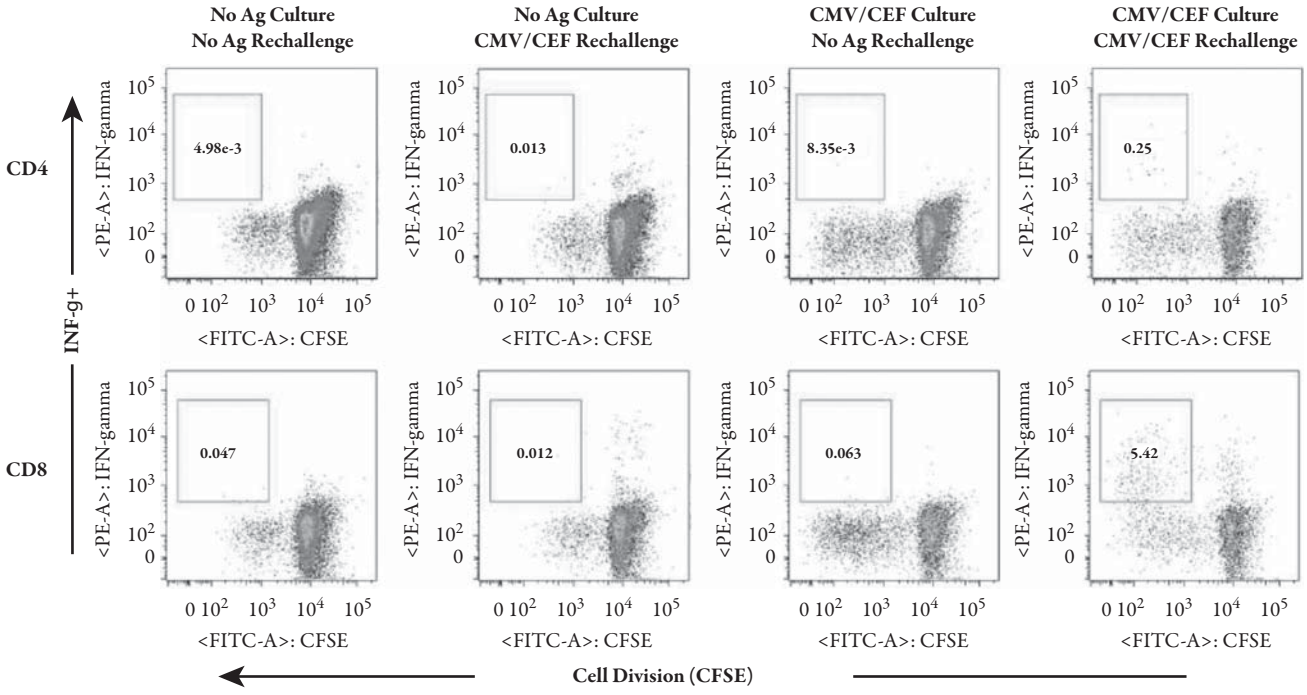

Figure 56.7 Multicolor (multiparameter) flow-cytometry analysis of cell division, surface-marker expression, and cytokine production. PBMCs from healthy cytomegalovirus (CMV)-immune adults were cultured under various conditions, with or without CMV, and with or without rechallenge with CMV at a later time point. These flow-cytometry dot plots show the fluorescence intensity of CFSE on the x-axis to measure cell division and staining with a monoclonal antibody against IFN-γ on the y-axis. In the upper panels, the analysis is restricted to ("gated on") cells expressing CD3 and CD4; in the lower panels, the plots are gated on cells expressing CD3 and CD8 (gating plots not shown). The dots inside each boxed region within each plot represent cells that have both undergone cell division and are expressing IFN-γ. This analysis shows that there are very few dividing and IFN-γ–expressing cells except under conditions where the cells are both incubated with CMV early and rechallenged later. Under these conditions, 0.25 percent of the CD4 cells and 5.42 percent of the CD8 cells divide and express IFN-γ. (Reproduced from Munier et al., 2009, with permission.)

type of assay in a clinical setting requires further study; it may not provide information that is equivalent to tritiated thymidine incorporation.

Apart from proliferation, there are many methods available to study distinct features of lymphocyte activation. Two broad categories include modulation of the expression of surface molecules and the production of cytokines. In general, the types of stimuli used are similar to those discussed above in the setting of the proliferation assay. Flow cytometry is well suited for both of these types of measurements. A variety of cell-surface markers have been used to monitor cellular activation, including CD69 (a C-type lectin rapidly upregulated in leukocytes after activation), CD25 (the α chain of the high-affinity IL-2 receptor), CD27 (a TNF receptor superfamily member expressed on activated and memory B lymphocytes), CD40 ligand (CD40L, expressed mainly only on $CD4^+$ cells), CD71 (transferrin receptor), and HLA-DR, among others (Fig. 56.8) (Last'ovicka et al., 2009; Pitsios et al., 2008). Any cytoplasmic cytokine of interest for which a suitable reagent is available may be studied by intracellular flow cytometry in fixed and permeabilized cells. Clearly, the multiparametric nature of flow cytometry is well suited to the simultaneous analysis of distinct lymphocyte subsets with respect to several surface markers and/or cytokines (Figs. 56.7 and 56.8).

Another type of T-cell–activation test is based on the production of ATP following stimulation with PHA (Hooper et al., 2005). This type of test is used widely in clinical organ transplantation and has defined predictive values with respect to the risk of infection and graft rejection. This test has not been widely applied for diagnosis of immunodeficiency.

As is the case with dye dilution methods, there are generally good correlations between surface marker or cytokine expression, and metabolic (ATP) measures of cellular activation with "traditional" testing based on tritiated thymidine incorporation. Although these different types of tests are intended to provide conceptually similar information regarding T-cell responsiveness to various stimuli, few have been subjected to a formal analysis of their predictive value in PIDD diagnosis. There may be clinically important differences in their interpretation as diagnostic tools. One small study indicates that CD69 expression may be a less-sensitive indicator of impaired T-cell function than tritiated thymidine incorporation (Hutchinson et al., 1999). Newer flow-cytometry–based methods require further additional evaluation in large patient cohorts to assess their performance as screening tools for diagnosis.

Cytotoxicity

Cytotoxicity describes the capacity of one cell to kill another cell by direct contact. $CD8^+$ T cells and NK cells are the two major, but not exclusive, cytotoxic cell populations. Therefore, tests concerning cytotoxicity focus on these populations. While cytotoxicity of $CD8^+$ T cells typically requires cognate interaction with the target cell, the license to kill of NK

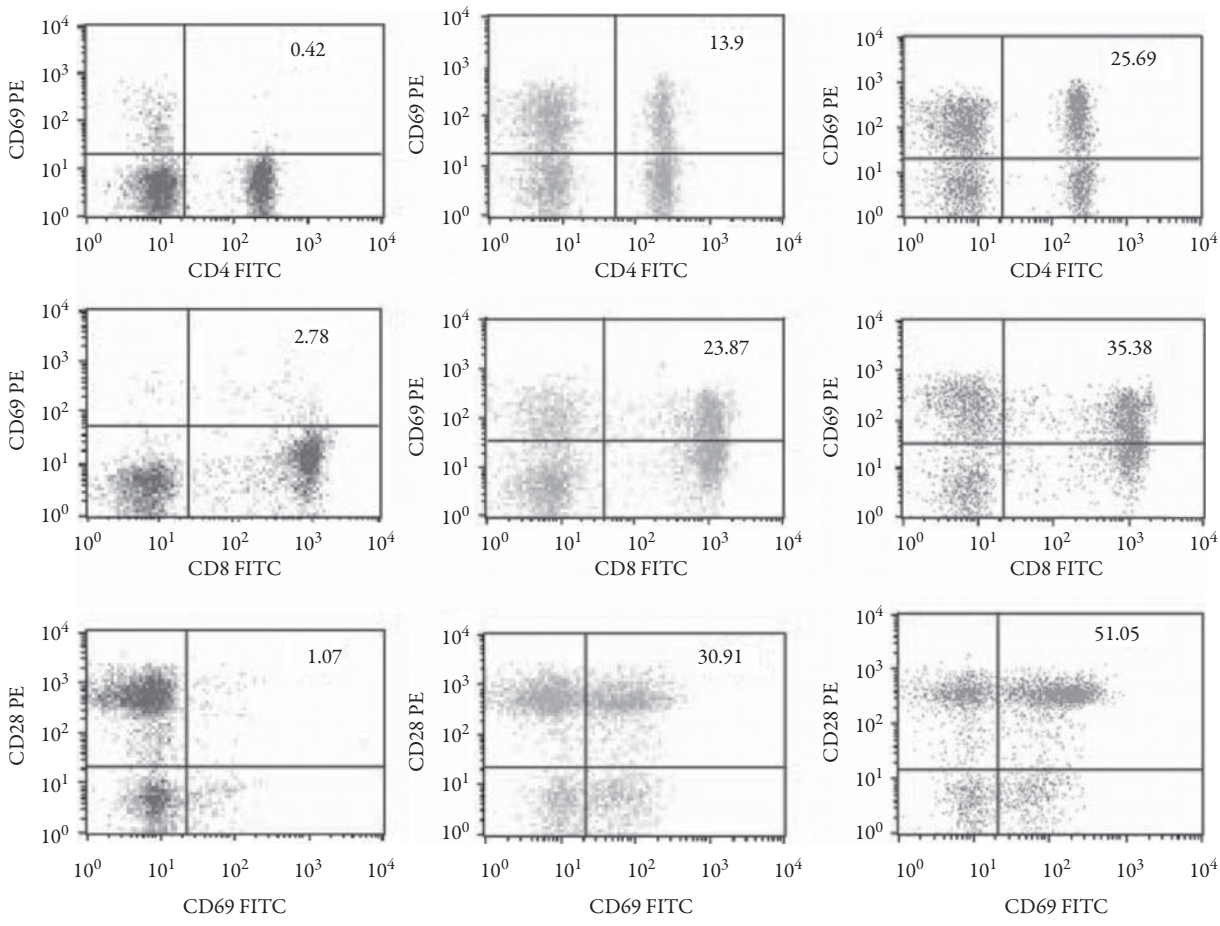

Figure 56.8 Multicolor (multiparameter) flow-cytometry analysis of lymphocyte activation by surface-marker expression. PBMCs from an HIV-infected adult were incubated without stimulation (left column of panels), with PHA (center column), or with a combination of monoclonal antibodies against CD3 and CD28 (right column). These dot plots represent the characteristics of T cells in the cultures. Plots are gated on cells that express CD3 (not shown). The top row of panels shows cells expressing both CD4 (x-axis) and the leukocyte activation surface marker CD69. The percentage of $CD4^+CD69^+$ cells is shown. The center row shows the results for $CD8^+$ cells; the bottom row shows expression of CD69 (x-axis) and CD28 (y-axis). (Reproduced from Pitsios et al., 2008, with permission.)

cells relies on less-specific activating and inhibitory signals. Cytotoxic cells kill through the release of cytotoxic granules (Fischer et al., 2007). Several disorders affecting the cytotoxic function present as hemophagocytic syndromes (Filipovich, 2008; see Chapter 54).

The standard assay for NK-cell function is the in vitro lysis of target cells. The target cell most often used is the erythroleukemia cell line K562. In the standard method, the K562 cells are loaded with ^{51}Cr. PBMCs of the patient, with or without activation with other agents such as PHA, are incubated with K562 tumor cells at various effector:target (E:T) ratios (von Zons et al., 1997). Cytotoxicity is measured after 4 hours of incubation by chromium release. While this radioactive assay is still the gold standard, several other nonradioactive assays for cytotoxicity have been developed (Allegra et al., 2006; Cholujova et al., 2008; Kim et al., 2007; Ozdemir et al., 2003). Their sensitivity and specificity in the context of immunodeficiency needs to be addressed in future studies.

In familial forms of hemophagocytic syndromes, NK-cell cytotoxicity is usually absent or markedly decreased in comparison to normal controls. The evaluation of the results needs to take into account whether NK cells are present and whether the function was tested during active disease or remission, since NK-cell function might normalize in secondary hemophagocytic syndromes and in X-linked lymphoproliferative syndromes (see Chapter 44). In the absence of NK cells in active hemophagocytic lymphohistiocytosis syndromes, CTL blasts can be generated in vitro and analyzed for cytotoxicity.

A flow-based assay to measure CD107 expression was suggested as a surrogate marker for the release of cytotoxic granules by $CD8^+$ T cells and NK cells (Betts et al., 2003). Further research needs to address whether this test can be used as a screening test for defects in cytotoxicity. In addition to functional assays, flow-cytometric evaluation of perforin might allow direct visualization before genetic or other molecular testing. Normal number but abnormal function of NK cells has also been reported in WAS (Orange et al., 2002) and Netherton syndrome (Renner et al., 2009).

Apoptosis

Disturbed apoptosis is the hallmark of ALPS (see Chapter 30). The majority of ALPS patients carry a mutation in the apoptosis-

inducing receptor FAS (CD95) (Su & Lenardo, 2008; Oliveira et al, 2010) and are designated ALPS-FAS. In addition to the increase of $CD4^-CD8^-$ double-negative $\alpha\beta$ TCR^+ T cells (DN T cells) to more than 2 percent, the essential diagnostic feature is the failure of induction of apoptosis of lymphocytes in vitro. Usually, PHA blasts are incubated with anti-FAS antibody or recombinant FAS ligand that is cross-linked to induce activation-induced cell death (AICD) (Rieux-Laucat et al., 1995). PBMCs are preferred to herpesvirus saimiri-transformed T cells or EBV-immortalized B-cell lines; even so, both are potential substitutes as targets (Broker et al., 1997). This assay will reveal about two thirds of the ALPS patients, including the FAS-deficient (ALPS-FAS) and caspase 10-deficient (ALPS-CASP10) patients (Su & Lenardo, 2008). The few Fas ligand (Fas-L)-deficient patients (ALPS-FASL) may be identified by the failure of their T-cell blasts to perform cytotoxicity in vitro (Del-Rey et al., 2006). In the subgroup of patients with somatic mutations of Fas (ALPS-sFAS), the decreased apoptosis and mutation can be seen only in sorted DN T cells (Holzelova et al., 2004) since the nonmutated T cells outgrow mutated T cells in culture and conceal the defective response of the DN T cells. The combination of DN T cells and elevated levels of soluble Fas-L, IL-10, and, surprisingly, vitamin B12 is a reliable marker of ALPS-FAS (Caminha et al., 2010; Magerus-Chatinet et al., 2009, Oliveira et al., 2010). Intrinsic defects of apoptosis can be tested by growth factor withdrawal; however, only a single patient has been described (Oliveira et al., 2007), and this is not part of routine diagnostic evaluation.

Signaling

Several PIDDs are characterized by disturbed leukocyte signaling. Defects can involve signaling via the B-cell receptor (e.g., XLA), T-cell receptor (ZAP70), TLR (IRAK4 or MyD88 deficiency), cytokine receptors (defects in STAT3, STAT1, or common γ chain, and others), or nuclear factor- κB (NF-κB) signaling (defects in NF-κB essential modulator [NEMO] or the inhibitor of κB kinase α chain). To date, mainly indirect functional assays have been performed to detect these deficiencies. With the increasing availability of phosphospecific antibodies, several of these immunodeficiencies can be analyzed by flow cytometry before the defect is confirmed by genetic analysis. Thus, recently Walshe et al. described the value of pSTAT5 detection by flow cytometry compared to Western blot and electrophoretic mobility shift assay (EMSA) in 32 SCID patients (Walshe et al., 2009). Absent STAT5 phosphorylation after IL-2 stimulation suggests defects in the common γ chain or in JAK3.

Bruton's tyrosine kinase (BTK) expression and phosphorylation can also be evaluated by flow cytometry. In patients without B cells, BTK expression can usually be determined in monocytes or platelets. This assay can also distinguish female carriers (Futatani et al., 1998). Defects in calcium signaling, as described in patients with mutations of Orai calcium release-activated calcium modulator 1 (ORAI1) (Feske et al., 2006) or stromal interaction molecule 1 (STIM1) (Picard et al., 2009), may be detected by the flow-cytometric measurement of the bound/unbound ratio of the calcium-sensitive dye Indo-1.

Mutations that permit protein expression but do not affect the phosphorylation site might not alter the flow-cytometric signal. Thus, pSTAT3-specific antibodies detect only STAT3-deficient hyper-IgE syndrome patients with mutations in the SH2 domain, but not in the DNA binding domain of STAT3 (Renner et al., 2008)

Radiosensitivity

Several PIDDs present with increased radiation sensitivity (Table 56.10). These are due to various DNA-repair defects (Chapters 46 to 49). To test for increased radiation sensitivity, usually cultured skin fibroblasts are irradiated with 1 to 4 Gy. Alternatively, EBV lines or immortalized T-cell lines of patients have been used. Sun et al. established normal ranges for a colony survival assay after radiation with 1 Gy (Sun et al., 2002). While only 13.1 ± 7.2 percent of the irradiated cells of patients with ataxia-telangiectasia (AT) survived after 24 hours, 50.1 ± 13.5 percent cells of healthy controls were still viable after the same treatment. The assay is time-consuming, since fibroblast or B- or T-cell lines need to be grown before the assay can be performed. Positive and negative control cultures have to be included in each experiment.

Recently, flow-cytometric assays have been developed using decreased phosphorylation of the histone γ-H2AX (Porcedda et al., 2009) or structural maintenance of chromosome 1 (SMC1) (Nahas et al., 2009), two phosphorylation targets of ATM that allow the identification of heterozygous carriers and homozygous AT patients. The great advantage of these assays is the rapid performance and the potential use of PBMCs. It remains controversial whether the count of γH2AX foci persisting after 24 hours is a reliable readout of increased radiation sensitivity (Markova et al., 2007).

Molecular Methods

Protein Detection

Where the clinical and screening laboratory phenotype is highly indicative of one or a few distinct possible genetic lesions, targeted molecular methods to analyze the gene product(s) may be employed. Labeled and unlabeled monoclonal antibodies for many molecules relevant for PIDDs are readily available from many commercial sources. Western blotting may be used to

Table 56.10 **SYNDROMES WITH INCREASED RADIATION SENSITIVITY**

Bloom syndrome
Ataxia-telangiectasia group
Ataxia-telangiectasia
Njimegen breakage syndrome
Ataxia-telangiectasia–like syndrome
S*CID phenotype*
Artemis deficiency
DNA ligase IV deficiency
Cernunnos deficiency

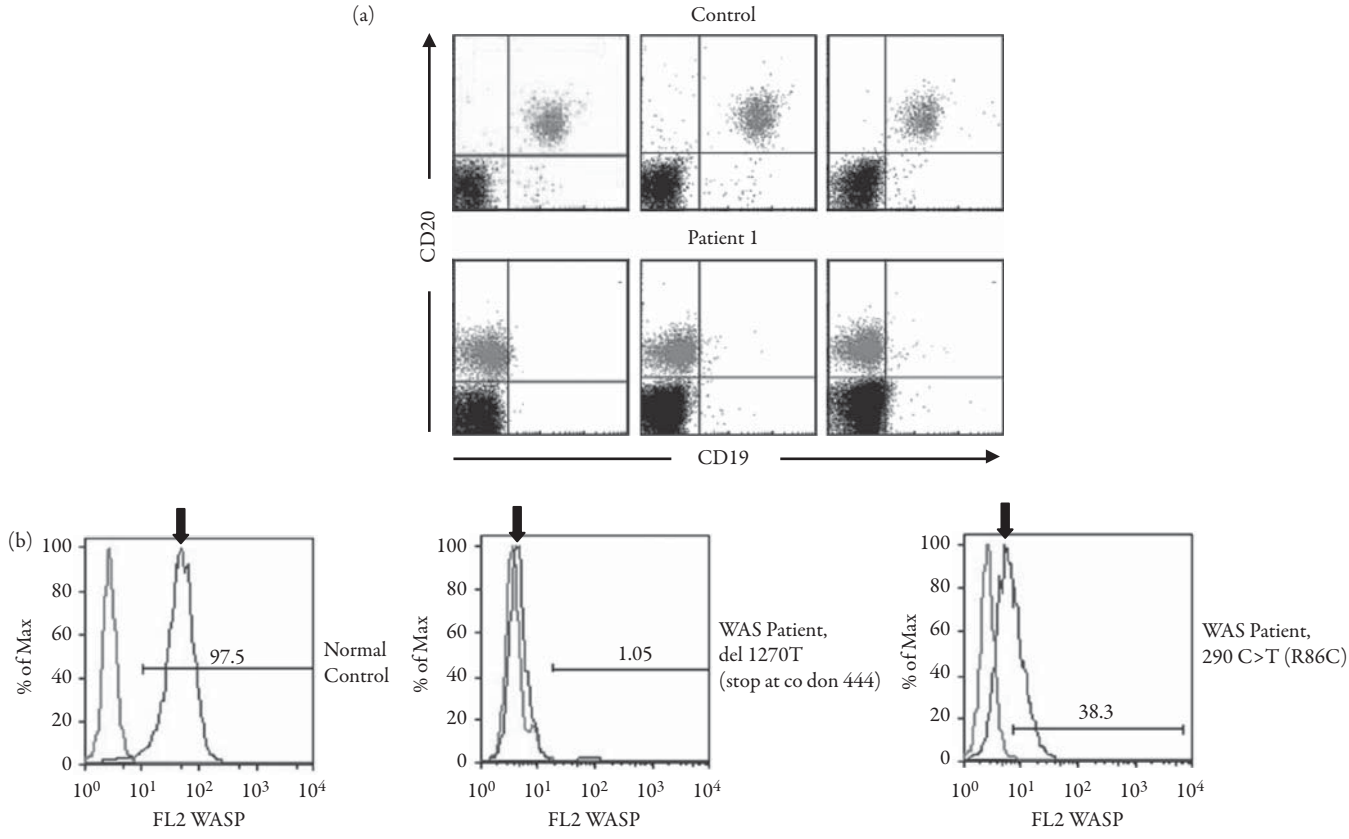

Figure 56.9 Flow-cytometric analysis of surface markers or cytoplasmic proteins for diagnosis of specific PIDDs. Part A: Peripheral blood lymphocytes are stained with monoclonal antibodies against CD20 (y-axis) and three different antibodies against CD19 (x-axis, three columns). A healthy adult control (top row) shows staining of B cells with all of the CD19 antibodies. A patient with CD19 deficiency has B cells (positive staining with anti-CD20), but these cells do not stain with any of the three anti-CD19 antibodies. (Reproduced from van Zelm et al., 2006, with permission.) Part B: PBMCs are permeabilized and stained with a monoclonal antibody against the Wiskott-Aldrich syndrome protein (WASP). The left panel shows the staining of a healthy adult control. The lymphocytes clearly show a shift in fluorescence with the anti-WASP antibody compared to the isotype control (left peak), indicating normal WASP expression. The middle and right panels show the staining patterns of two patients with WAS having two different WASP mutations. The middle panel shows a patient with a deletion leading to premature termination of translation and absence of WASP. The right panel shows a patient with a missense mutation that results in low-level expression of the mutant WASP. The low level of staining could be due to intrinsic instability of the mRNA and/or the protein, reduced binding of the staining antibody to the mutant WASP, or some combination of these factors. (Reproduced from Ochs & Thrasher, 2006, with permission.)

detect the presence, quantity, or size of any protein for which a suitable antibody is available (Becker-Catania et al., 2000; Holinski-Feder et al., 1998; Jirapongsananuruk et al., 2003; Lemahieu et al., 1999). Overall, this method is relatively cumbersome in comparison to methods based on flow cytometry but can be particularly useful for detecting altered structural forms, particularly those that are smaller or larger as a result of mutation. Flow cytometry may be used to study cell-surface, cytoplasmic, and nuclear molecules. The latter two require permeabilization of cells prior to addition of labeled antibodies. Examples include CD3ε (on T cells), CD19 (on B cells), CD25 (on T cells or B-cell lines), CD40 (on B cells), CD40L (on activated T cells), inducible T-cell co-stimulator (ICOS) (on activated T cells), BTK (in monocytes or platelets), WASP (in lymphocytes or monocytes) (Nakajima et al., 2009), SLAM-associated protein (SAP) by activated T cells (Shinozaki et al., 2002), and BAFFR on B cells (Warnatz et al., 2009). Some examples are shown in Figure 56.9. Point mutations may lead to dysfunction but may not alter expression, and therefore normal flow-cytometric detection of the suspected molecule may be misleading.

RNA Detection

A variety of molecular methods are available for the study of single gene mRNA expression and structure for diagnostic purposes (see Chapters 2 and 57). These include Northern blots, dot blots, and PCR methods (including quantitative RT-PCR), alone or in conjunction with single-strand conformation polymorphism analysis. (Holinski-Feder et al., 1998; Lemahieu et al., 1999)

Gene Structure

Methods for the study of gene structure are increasingly applied for the diagnosis of PIDDs. These include methods such as Southern blotting to detect large-scale changes (deletions), as well as genomic sequencing to identify smaller mutations. Demonstration of altered genetic structure is necessary but not sufficient for establishing a definitive PIDD diagnosis. Particularly in the case of new missense mutations, there must be plausible or clearly demonstrated functional alteration of the gene product as a result of the mutation, as well as absence of the mutation in the general population. Genetic aspects of PIDDs are discussed in Chapters 2 and 57.

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57.

GENETIC ASPECTS OF PRIMARY IMMUNODEFICIENCIES

Jennifer M. Puck

There are three reasons why evaluation of an immunological disorder in any patient should include consideration of potential underlying genetic determinants. First, genetic studies can confirm a suspected diagnosis of immunodeficiency and indicate the best treatment; they can also help to pinpoint a specific primary etiology even when other available data are unusual, incomplete, or inconclusive. Second, new understanding of the immunological basis and pathogenesis of immunodeficiencies can be gleaned as more disease genes are identified through the partnership between researchers in molecular genetics, clinicians, and affected patients. Finally, diagnosing a genetic disorder in an index patient, or proband, has profound implications for the patient's family members, both affected and unaffected, living and as yet unborn.

Disorders of the immune system may be due to defects in single genes, either already known or currently unidentified, that encode gene products essential for development or function of immune pathways. Because we have now recognized newly arising mutations and variant phenotypes for many immunodeficiency genes, we know that even immunodeficient patients who lack a positive family history may have genetic mutations underlying their condition that pose a risk of recurrence in their offspring or other family members.

More common on a population basis than single gene disorders causing immunodeficiency are many as yet incompletely understood immune dysfunctions with a complex etiology with one or more genetic factors that may act in conjunction with environmental influences. As the fields of genetics and genomics advance, genetic contributions to all categories of human disease, including immunodeficiencies, become increasingly apparent. While this chapter emphasizes the available genetic tests for single gene diseases, the field is changing rapidly and promises to be radically different when whole genome sequencing becomes widely available. Not only are additional disease genes being discovered, but technologies are improving to understand the significance of observed gene variants. The immense increases in technical ability to determine an individual's genetic constitution present a formidable challenge to our medical and social systems to help individuals understand their risks, and to develop new therapies based on genetic advances.

The reader is referred to Chapter 2 for a summary of genetic principles and technologies. This chapter will present an overview of genetic tests used to diagnose inherited immunodeficiencies, but these tests should be viewed in a broad context of evolving basic knowledge, technological advances, and social and regulatory issues. The physician specializing in the diagnosis and treatment of immunodeficiencies should stay in contact with patients and their families to be able to pass on relevant new information. Much can be gained through enlisting the involvement of clinical geneticists and genetic counselors, who specialize in helping individuals and families grasp and manage their personal genetic risks.

ESTABLISHING A GENETIC ETIOLOGY

POSITIVE FAMILY HISTORY

The first diagnostic measure when considering any heritable disorder is to obtain a full pedigree and family health history; heritable immunodeficiencies are no exception. Although most of the immunodeficiencies in this book have been identified at the gene level recently, families can often provide details about potentially affected members in past generations whose presentation and course might be compatible with the disorder (see agammaglobulinemia pedigree and discussion in

Table 57.1 X-LINKED IMMUNODEFICIENCIES AND THEIR DISEASE GENES

IMMUNODEFICIENCY DISEASE	GENE NAME	PROTEIN	LOCATION	FEMALE SKEWED X INACTIVATION	AUTOSOMAL GENOCOPY	SEE CH
X-linked chronic granulomatous disease (CGD)	*CYBB*	Cytochrome oxidase gp91^{phox} that generates reactive oxygen species	Xp21.1	No	Yes	51
Properdin deficiency	*PFC*	Properdin activator of complement	Xp11.23	No	Yes	54
Immunodysregulation polyendocrinopathy enteropathy sundrome (IPEX)	*FOXP3*	Forkhead/winged-helix transcriptional regulator critical for regulatory T cells	Xp11.23	No	Not known	31
Wiskott-Aldrich syndrome (WAS)	*WAS*	WAS protein (WASP) activator of actin polymerization and signal transduction	Xp11.22	Yes: all hematopoietic cells	Yes	42
X-linked severe combined immunodeficiency (XSCID) Immunodeficiency X-linked with magnesium transport defect (XMEN)	*IL2RG* *MAGT1*	Common γ chain of cytokine receptors Magnesium transport protein; defect leads to severe Epstein-Barr virus infections, lymphoma	Xq13.1 Xq21.1	Yes: T, B, NK cells (not phagocytes) Yes: T cells	Yes Not known	10 (Li et al., 2011)
X-linked agammaglobulinemia (XLA)	*BTK*	B cell tyrosine kinase	Xq21.3-q22	Yes: B cells (not T cells, phagocytes)	Yes	24
X-linked lymphoproliferative syndrome type 2 (XLP2) X-linked lymphoproliferative syndrome type 1 (XLP1)	*XIAP* *SAP, SH2D1A*	Inhibitor of apoptosis XIAP Lymphocyte signaling activation molecule	Xq25 Xp25	Unknown Yes: NKT cells (not T, B cells)	Yes Yes	43 43
X-linked hyper-IgM syndrome	*CD40L*	CD40 ligand	Xq26.3-q27.1	No	Yes	25
G6PD deficiency	*G6PD*	Glucose-6-phosphate dehydrogenase	Xq28	No	No	49
X-linked ectodermal dysplasia with immunodeficiency (NEMO) Dyskeratosis congenita X-linked hypogammaglobulinemia with growth hormone deficiency	*NEMO, IKBKG* *DKC1* *[ELF4?]*	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase γ Component of telomerase; defect causes premature aging, myelodysplasia (Myeloid ELF-like factor [MEF])	Xq28 Xq28 [Xq26? Not proven]	Sometimes; females may have incontinentia pigmenti Yes (in all cells in a mouse model) Yes: B cells	Not known Yes Not known	35 (He et al., 1998) 24

Chapter 1). Assembly and recall of complete information often requires more than one session and interviews with more individuals than just the nuclear family of the index case. Retrieval and review of medical records from relatives can provide critical data, so they are well worth seeking. Ethnic and geographical ancestry should be recorded as part of every family history because they may suggest consanguinity or increased risks associated with particular populations.

The X chromosome harbors a large number of recognized immunodeficiency genes, and the X-linked immunodeficiency diseases occur in disproportionately high frequency (Table 57.1). This is because in males, mutations in genes on the sole copy of the X chromosome are uncompensated, and therefore a single mutation causes overt disease, while females with one mutated and one normal gene copy generally remain healthy. The X-linked inheritance pattern of multiple

affected males related through maternal lines, as discussed in Chapter 2, should be sought in all families with a male presenting with immunodeficiency.

Autosomal recessive diseases, in contrast, require defects in both alleles of a gene for a disease phenotype to be manifest. One way in which this occurs is through consanguineous marriages. Another risk factor for homozygous recessive diseases is belonging to a genetically isolated population, in which the same mutation may be transmitted through both maternal and paternal lines to the patient from a common ancestor. A rare cause of homozygous recessive inheritance is isodisomy, in which an affected individual has inherited both copies of a chromosome from one parent, rather than one copy from each parent. However, in most recessive immunodeficiencies, such as adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID) (Chapter 14), patients are predominantly compound heterozygotes, having a different mutation in each of their two alleles.

There are relatively few dominant immunodeficiency phenotypes. As a rule they are characterized by incomplete penetrance (skipping generations in family pedigrees) and/or variable expressivity (a large spectrum of severity among relatives with the same genetic mutation). Examples include DiGeorge syndrome (Chapter 45); autoimmune lymphoproliferative syndrome due to Fas deficiency (Chapter 30); some instances of hyper IgE-recurrent infection syndrome or Job syndrome (Chapter 38); and, importantly, some instances of common variable immunodeficiency and IgA deficiency (Chapter 28) and even atopy and allergic disorders (Holgate, 1997). These conditions may turn out to be diseases in which a dominant gene mutation has a predisposing or contributory effect, but in which genetic or environmental factors act as modifiers of the phenotype.

Several immunodeficiency syndromes, such as SCID or chronic granulomatous disease (CGD), can be caused by defects in any of several different genes; the existence of autosomal phenocopies of the X-linked immune disorders is indicated in Table 57.1. Family history can be critical in differentiating the X-linked from autosomal forms.

IMPORTANCE OF FAMILY HISTORY, EVEN IF NEGATIVE

Documentation of a complete family pedigree is helpful even if it fails to reveal any sign of disease comparable to that of a proband affected with immunodeficiency. First, the structure of the pedigree determines the quantity of genetic information. A large kindred with many siblings of both sexes has had more opportunities to have affected relatives, whereas at the other extreme, there may be no information whatsoever about an adopted proband's heredity. Second, the absence of disease in many relatives who could potentially be at risk is useful. For example, if a male with SCID has many healthy maternal uncles, either a new occurrence of an *IL2RG* gene mutation (Table 57.1) or recessive disease is more likely than an X-linked mutation inherited from the patient's grandmother. A third benefit of obtaining a family history is the insight it can provide into the way in which family members will approach a serious immunodeficiency disease. We can provide optimal care for patients and their families only if we understand what the disease means to them. In previous generations or when the initial diagnosis has been delayed and treatment was ineffective, children born with immunodeficiencies are likely to have died, leading to parental feelings of guilt, secrecy, and other family stresses. Important family events or beliefs unrelated to immunodeficiency also strongly influence the outlook of family members. Finally, the family history may bring to light facts that influence the type of genetic testing that may be indicated, such as multiple miscarriages (a risk factor for chromosomal rearrangements) or misattributed paternity.

GENETIC TESTING FOR ABNORMALITIES IN IMMUNE SYSTEM GENES

Inherited immunodeficiencies are sufficiently rare that population-based screening would be required to make the diagnosis prior to the onset of infectious or autoimmune complications; screening has become possible for SCID (see below). Genetic testing is otherwise used to establish the diagnosis in an affected individual or to determine the status of relatives of a person already suspected to have a primary immunodeficiency. Individuals in a family with recognized immunodeficiency for whom genetic testing may be indicated include affected patients who are ill; individuals who may have inherited an defective immune system gene but as yet show no symptoms (presymptomatic testing); and healthy relatives who are not at risk for becoming immunodeficient themselves but who may be carriers of a defective copy of a gene and are concerned about their reproductive risks. In addition, prenatal testing has been performed for many immunodeficiencies in pregnancies that occur subsequent to the diagnosis of an affected proband. No single testing modality is appropriate for all situations. The reader is referred to specific chapters for discussions of the options for each disease.

TECHNIQUES THAT DO NOT RELY ON ISOLATION OF PATIENT DNA OR RNA

BIOCHEMICAL TESTS

The immunodeficiencies associated with enzyme abnormalities such as ADA and purine nucleoside phosphorylase (PNP) deficiency can be diagnosed by detecting increased amounts of physiological precursors, dATP or dGTP, respectively, or by direct measurement of enzyme activity in vitro (see Chapter 14). Similarly, glucose-6-phosphate dehydrogenase (G6PD) deficiency, associated with impaired intracellular killing by neutrophils, is best detected by enzyme assay (Beutler et al., 1979; Luzzatto & Mehta, 1995). The advantage of biochemical testing is that the functional consequences of the underlying gene mutation can be immediately demonstrated without the need for DNA sequencing. Moreover, gene defects that permit production of inactive or partially active protein will be distinguished from DNA sequence variants

of no physiological significance (genetic polymorphisms). A disadvantage, however, is that carrier testing, which relies on demonstrating enzyme activity intermediate between the normal and deficient ranges, may be imprecise due to overlap with the partial activity of some mutant proteins in affected individuals. Careful assay standards with positive and negative controls in every run are essential.

IMMUNOLOGICAL TESTS

Enumeration of leukocyte populations; measurement of their secreted products, such as immunoglobulins; and functional tests involving in vitro activation are necessary for the definitive diagnosis of virtually all immunodeficiencies. Their use in diagnosis is discussed in Chapter 56. In only a few circumstances, however, have these immunological tests been made specific for a particular gene product so that they can be used to establish a specific gene diagnosis. One example is X-linked hyper-IgM syndrome due to CD40 ligand (CD40L) deficiency (Table 57.1 and Chapter 26). Stimulated T cells from healthy individuals express CD40L, which can be detected by binding to immunofluorescent-tagged soluble CD40 or to an anti-CD40L monoclonal antibody (Conley et al., 1994). However, mutations that permit production and expression of defective protein at the cell surface may not be detected by this test, and it may be unreliable for the testing of female heterozygous carriers and cannot be used for prenatal testing early in pregnancy.

Another example of an immunodeficiency with a useful immunological test is CGD (Chapter 52). Formerly diagnosed by counting cells able to reduce nitroblue tetrazolium dye, the inability of CGD granulocytes to produce superoxide is now most accurately measured by flow-cytometric determination of neutrophil NADP oxidase activity (Jirapongsananuruk et al., 2002). Not only are patient cells readily shown to be defective in this test, but the results can be quantified to yield predictive information about disease severity. Moreover, female carriers of the X-linked form of CGD can be identified because the Lyonization, or random X inactivation, of their neutrophils results in two distinct populations when their cells are analyzed (see below). Cells whose active X chromosome has an intact *CYBB* gene have normal NADP oxidase, while those with an active X chromosome bearing mutated *CYBB* demonstrate the same lack of activity as the affected proband.

A special use of immunological tests is in the prenatal evaluation of fetal blood samples. By the middle of the second trimester of pregnancy it is possible to obtain blood from the fetal umbilical vein to determine whether functional T cells, B cells, or granulocytes are present in normal numbers (Lau & Levinski, 1988). Fetal blood sampling involves greater risk to the fetus than does either amniocentesis or chorionic villus biopsy, and it should be performed only in a center with extensive experience in the technique. It is possible for the sample to be contaminated with maternal blood. Only a small volume of fetal blood is collected, and it must be analyzed without delay by an immunology laboratory that has established normal fetal blood ranges. Despite these caveats, fetal blood sampling is undertaken by certain pregnant couples who have had a previous affected child with immunodeficiency but who lack a gene-specific diagnosis. For example, cell-surface staining for T cells and lymphocyte mitogen responses can be tested in a fetal blood sample to rule in or rule out a diagnosis of SCID in a pregnancy at risk, even if the gene mutation causing SCID in the proband is not known.

CYTOGENETIC STUDIES

Cytogenetic studies involve the microscopic analysis of chromosomes, usually when they are condensed during metaphase of mitosis. Giemsa banded karyotypes can reveal abnormal numbers of chromosomes or large structural changes, such as translocations, deletions, or duplications. The most common cytogenetic abnormality, trisomy 21 or Down syndrome, is associated with many qualitative immune defects but is almost always diagnosed because of its physical stigmata before immune dysfunction is recognized. While chromosomal abnormalities should be suspected when immune dysfunction is accompanied by additional congenital anomalies, there are instances in which a cytogenetic abnormality has only an immune phenotype, such as in males with X-linked lymphoproliferative disease due to deletions within Xq25 (Chapter 44). Pursuit of cytogenetic abnormalities has led directly to the identification of at least one immunodeficiency gene, *CYBB* in X-linked CGD (Orkin, 1989). A small minority of CGD patients have deletions of *CYBB* large enough to be detected cytogenetically. Patients with these deletions often have deletion of multiple contiguous genes, leading to a phenotype of CGD plus other abnormalities (Chapter 52). Balanced translocations can be clinically silent except for the disruption of particular genes at the chromosomal breakpoints, but a family history of multiple miscarriages should prompt cytogenetic investigation because offspring undergoing fetal demise may have received an unbalanced parental chromosomal complement. Thus, while the yield of karyotype analysis may be rather low in immunodeficiencies, this simple test is at times extraordinarily helpful in uncovering a genetic basis for a puzzling clinical syndrome and pinpointing the region of the genome involved. One caveat is that cytogenetic analysis is generally done on phytohemagglutinin-stimulated peripheral blood samples; if lymphocytes are present in low numbers and have impaired function, they may fail to respond to mitogen activation, leading to inadequate mitoses for scoring.

The power of cytogenetics was extended by fluorescent in situ hybridization (FISH). In this technique, a cloned unique human DNA fragment is tagged with a fluorescent label and allowed to hybridize to its corresponding sequence within chromosomes spread on a microscope slide. Viewed with a fluorescence microscope, the two homologous chromosomes are marked with the fluorescent DNA probe. FISH can be used to diagnose interstitial deletions on one copy of chromosome 22q11 associated with DiGeorge syndrome (thymic hypoplasia, hypoparathyroidism, and conotruncal heart malformations; Chapter 45). Although some deletions are large enough to be detected by karyotype analysis, especially with high-resolution technique, FISH with fluorescent probes

from 22q11 reveals only one signal in 90 percent of DiGeorge syndrome patients (Color Plate 45.I or 45.II).

A copy number array is a new DNA-based technique (see below) that many now prefer over karyotype or FISH because it provides information about deletions and insertions throughout the genome, although it does not reveal balanced translocations.

DNA- AND RNA-BASED TECHNIQUES

Molecular methods for mutation diagnosis are being applied to thousands of genetic conditions today, and many more genetic tests, including whole genome sequencing, will be common in the future. Certain generalizations distinguish mutations underlying immunodeficiency genes from those causing many other well-studied diseases. In some disorders, such as sickle cell disease, a single mutation predominates (Kan, 1992). Even in the cystic fibrosis disease gene CFTR, about two thirds of mutated chromosomes in Caucasians have the same Δ508 mutation (Zelenski & Tsui, 1995), and testing for a panel of mutations can effectively diagnose the great majority of cases (Richards et al., 2002). In contrast, immunodeficiency gene mutations tend to be extremely diverse, with half or more of the mutations unique to a single family. Unlike the dystrophin gene, defective in Duchene muscular dystrophy, in which large deletions and duplications account for 66 percent of mutations (Worton, 1992), by far the most common immunodeficiency gene mutations are small changes on the order of one to a few nucleotides, occurring in both coding sequences and their adjoining splice regions. An important new class of mutations described in human neurological and muscular diseases is the expansion of triplet repeats either in coding regions or in regions near a gene that are subject to silencing by methylation (Everett & Wood, 2004). However, to date no immunodeficiency-causing gene mutation of this type has been described.

In other respects, gene mutations that cause inherited immune disorders are similar to those in other genetic diseases (discussed in Chapter 2). These include a high proportion, approximately one third, of newly arising mutations for X-linked disorders (Haldane, 1935). As previously noted for the hemoglobin locus, factor IX locus, and many other genes (Cooper & Krawczak, 1995), the occurrence of C-to-T mutations at CpG dinucleotides in immunodeficiency genes is greatly increased above the number that would be expected if all nucleotide changes were completely random.

LINKAGE TO PCR-BASED POLYMORPHIC MARKERS

Linkage analysis is discussed in some detail in Chapter 2. For immunodeficiency diseases mapped to a unique genomic region but not yet identified, linkage analysis can define which chromosome segments in a family carry mutated disease gene alleles, provided there are both affected and unaffected relatives available for testing. Linkage analysis can also be useful when the gene that is defective in a family is known, but the specific mutation has not been determined. Linkage diagnosis can be used for carrier testing and for prenatal diagnosis. One should select tightly linked markers that flank the disease locus and are polymorphic in the required members of the family being tested. A haplotype of flanking marker loci is determined for disease-bearing and normal chromosomes in the parents of the individual at risk. If the flanking alleles on both sides of the disease locus match parental alleles from an unaffected chromosome, the person is likely to have inherited the entire haplotype block of DNA, including the normal copy of the disease gene. Similarly, flanking alleles co-inherited from a disease-bearing chromosome predict that a mutated copy of the disease gene has been inherited (see Chapter 2).

The first linkage markers to be used were restriction fragment length polymorphisms (RFLPs), nucleotide variations resulting in the presence or absence of a restriction enzyme site at a defined genomic DNA position (Botstein et al., 1980; Drayna & White, 1985). Digestion of genomic DNA with the relevant restriction endonuclease will demonstrate allelic variants with either shorter or longer DNA fragment sizes depending on whether the polymorphic restriction site is present or absent. Fragment lengths are assayed by Southern blotting.

The discovery of PCR revolutionized linkage analysis. PCR depends on designing short synthetic oligonucleotides flanking a DNA segment to be amplified. These oligonucleotides, called primers, are added in excess to genomic DNA along with free nucleotides, magnesium salts, pH buffer, and a heat-stable DNA polymerase enzyme, such as Taq polymerase. First, the reaction mixture is denatured by heating to 95°C so that the DNA helices unwind and the DNA becomes single-stranded. Next, the mixture is cooled to a moderate temperature, typically between 50° and 65°C, enabling the oligonucleotides to bind by complementary base pairing to the particular regions of template DNA that they exactly match. Finally, warming the mixture to 72°C allows the polymerase to synthesize new DNA using the oligonucleotides as primers and adding free nucleotides to form new complementary strands. Each repetition of this sequence of denaturation, annealing, and extension produces a doubling of the quantity of each template strand. This succession of temperatures constitutes one cycle of PCR. In successive cycles the segment of DNA between the primers is exponentially amplified. Eventually the amount of product becomes large enough to detect by direct visualization after gel electrophoresis.

Segments of DNA that are polymorphic can be readily detected by PCR. If the primers flank a polymorphic restriction site, the product can be digested to assign the restriction site allele. Another type of polymorphism is called a microsatellite or short tandem repeat polymorphism (STRP). The most common repeated unit, occurring on average once every 50,000 nucleotides throughout the genome, is CA on one DNA strand, GT on the opposite strand (Litt & Luty, 1989; Weber & May, 1989). Other repeats of units of two to five nucleotides also occur frequently. Very often, assorted numbers of repeated units are found in chromosomes from different individuals, and a stable number of repeats is inherited from parents to offspring. PCR amplification of the DNA segment

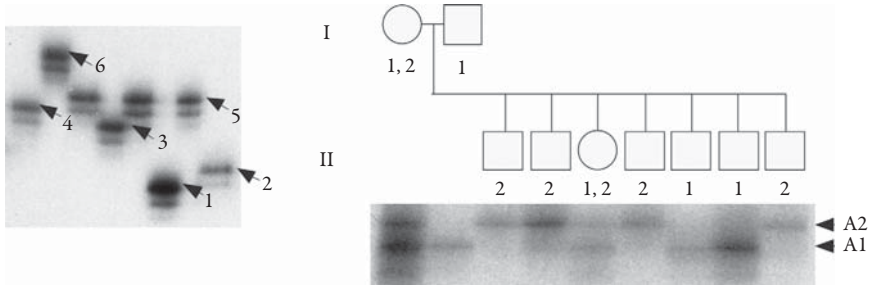

Figure 57.1 Short tandem repeat polymorphism (STRP). Right, DNA from eight unrelated males amplified with primers defining STRP locus DXS441 in Xq13 (Ram et al., 1992). Six alleles, numbered according to their increasing numbers of CA repeat units, were amplified using flanking PCR primers. Darker bands are the primary signal; lighter "shadow" bands seen below each primary band are typical artifacts in CA repeat STRPs. Left, DNA from family members amplified with primers defining the STRP GATA182e04 at Xq28.

containing a series of simple repeated units, followed by size separation by gel electrophoresis, reveals the length differences between the polymorphic alleles. Each lane of the gel on the left of Figure 57.1 shows DNA from an unrelated male amplified with appropriate primers surrounding the CA repeat present at the STRP marker DXS441 in Xq13, close to the X-linked SCID locus (Ram et al., 1992). Alleles of six distinct sizes are seen, indicating that the number of CA units can vary considerably between individual X chromosomes. The right side of the figure shows inheritance of a different X-linked STRP, GATA182e04, in a kindred. The parents share allele A1 and the mother is heterozygous for this allele and A2. The allele content of offspring is shown below their symbols: the sons are each hemizygous for one of the maternal alleles while the daughter is heterozygous, carrying paternally derived A1 and maternally derived A2.

STRPs have several advantages compared to RFLPs, reflecting the power of PCR as a tool for molecular genetic analysis. Foremost is the high frequency of STRPs throughout the genome. For linkage studies, STRPs can be found near any immunodeficiency gene. STRPs are also easily assayed by PCR. Miniscule amounts of input DNA template can be successfully amplified, including DNA recovered from newborn screening dried blood spots, formalin-fixed tissues, or histological slides. Thus, genetic information may be derived even if an affected patient has died without having donated samples for molecular analysis. Finally, the multiplicity of commonly observed alleles means that it is likely that a child's alleles can be unambiguously traced to one or the other parent.

Even more common than STRPs are single nucleotide polymorphisms, or SNPs, which occur at a rate of around one per 1,000 nucleotides of genomic DNA sequence. Unlike highly informative STRPs, SNPs have only two alleles, but their huge numbers make up for this inconvenience; the database dbSNP at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov) contains over 50 million SNPs with a minor allele frequency of at least 5 percent. Clusters of neighboring SNPs tend to be inherited together in haplotype blocks that have been maintained with little crossing over since ancestral origins of humans (Gabriel et al., 2002), so that SNP signatures can identify ancestral haplotype blocks to streamline linkage analysis.

COPY NUMBER ARRAYS

Copy number array testing is a high-throughput method for detecting both the presence and the intensity of signals hybridizing to a large number of specific oligonucleotides throughout the genome, including polymorphic SNPs. SNP content (for example A/A, A/C, or C/C at a given position) and intensity of the signal relative to neighboring and distant signals is detected, making possible identification of haplotypes and revealing interstitial deletions or duplications (copy number variations [CNVs]). Many CNVs are recurrent while others are unique and of unknown significance. However, in the context of clinical findings or additional genetic information, CNVs can explain phenotypes. For example, Figure 57.2 shows a copy number analysis of proximal chromosome 16p11.2 in DNA from a patient with

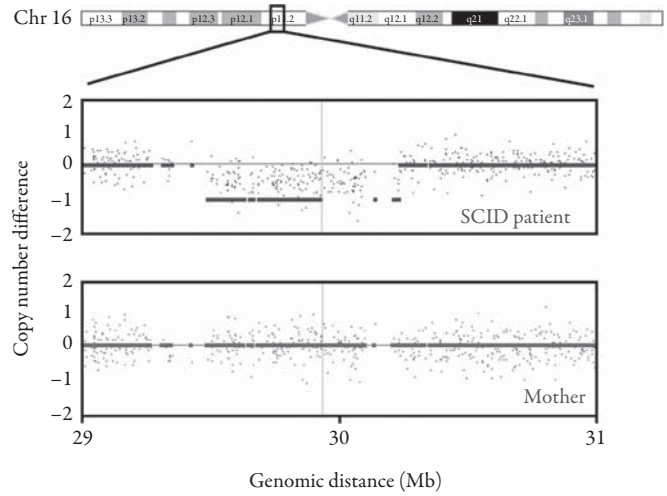

Figure 57.2 Copy number array analysis in a SCID patient and her mother across a 2 MB region of chromosome 16p11.2. Patient hybridization intensity is approximately half of normal (copy number -1) over a 600 bp region, diagnosing an interstitial deletion of one of her two copies of chromosome 16. The mother does not have a deletion in this region.

SCID (top) and her mother (bottom). The patient has a 600 kb interstitial deletion, indicated as "-1" copy number compared to her adjacent regions of diploid signal strength. The patient is hemizygous for over 24 genes in the deleted region. One of the deleted genes, CORO1A, encoding Coronin-1A, a lymphocyte protein involved in actin polymerization and cell motility, was also mutated in her paternally derived allele, resulting in an atypical SCID phenotype (Chapter 23, Shiow et al., 2008). Moreover, the region is now appreciated to be a recurrent CNV, prone to deletions and duplications because of crossing over of the direct repeats at each end; in fact, the patient's deletion was de novo, occurring on her maternally derived allele, but not found in her mother's blood cells. Other genes in the region currently under study are associated with infantile seizures (PRRT2), attention-deficit/hyperactivity disorder, and autism spectrum disorder when present in abnormal copy number (deVries et al., 2012; Luo et al., 2012; Weiss et al., 2008).

SOUTHERN BLOTTING, EVALUATION OF GENOMIC DNA

Southern blotting, named for its inventor, E.M. Southern (1975), is the prototypic technique for specific gene detection. A cloned segment of DNA is synthesized to incorporate labeled nucleotides, such as radioactive ^{32}P, and this labeled DNA is used as a probe to hybridize to a complex mixture of DNA, such as restriction enzyme digested genomic DNA prepared from the cells of a particular person. Now considered too labor-intensive for most clinical applications, this was the method historically used for the evaluation of RFLP markers and changes in genomic DNA before the development of PCR. Southern blotting can detect disease-causing mutations due to either large DNA deletions or rearrangements affecting the disease gene or due to single nucleotide changes that alter restriction enzyme sites. Southern blot variations have often provided the first clue that a candidate gene in fact harbors deleterious mutations in individuals with inherited immunodeficiencies. For example, they were used to find some of the first BTK mutations in patients with XLA (Vetrie et al., 1993). However, because the great majority of immunodeficiency gene mutations are small changes of one to a few nucleotides, Southern blot analysis is insensitive for finding suspected mutations in new patients. Moreover, finding altered Southern blot band patterns does not directly indicate the location or specific nature of a mutation. However, in families with a previously documented disease-associated Southern blot abnormality, this method can be successfully applied for carrier and prenatal diagnosis in relatives at risk of having inherited the same defect.

NORTHERN BLOTTING AND REVERSE TRANSCRIPTASE-PCR

Many mutations within or adjacent to exons of genes affect the quantity or size of the mRNA produced. These changes can be detected by Northern blot analysis, which is carried out in a manner similar to that of Southern blots. RNA is isolated from cells and electrophoresed through a gel to achieve separation by size. After transferring the RNA to a membrane, a labeled nucleic acid probe is allowed to hybridize to it, identifying a band of RNA containing the specific nucleotide sequence of interest. Mutations can produce differences in expected size or amount of RNA detected by Northern analysis.

As with DNA analysis, PCR has greatly enhanced our ability to analyze RNA through a process called reverse transcriptase PCR (rt-PCR). First, a complementary DNA (cDNA) strand is created by incubating mRNA with the enzyme reverse transcriptase and a DNA oligonucleotide primer. Even the nanogram quantities of RNA that can be purified from small numbers of cells can yield enough cDNA for successful amplification by PCR. Amplified cDNA can be sequenced, sized by gel electrophoresis, and quantitated to reflect the amount of cellular mRNA originally present (provided carefully monitored conditions and appropriate controls are used).

Mutations that disrupt the 3′ and 5′ splice motifs that flank exons prevent normal processing of newly made mRNA. These mutations are generally associated with low or undetectable levels of correctly spliced mRNA in the cytoplasm of cells. Aberrantly spliced mRNA species may be detected, such as RNA retaining an intron that would normally be excised. Missense or frameshift mutations that generate termination codons within the 5′ portions of coding sequences also usually cause mRNA to be degraded (known as nonsense-mediated decay). Thus, studies of mRNA are important in assessing the actual consequences of any mutation observed in DNA sequence. However, investigation of mRNA quantity or sequence is often complex and therefore not well suited to clinical diagnostic testing.

X-CHROMOSOME INACTIVATION PATTERN INPOTENTIAL FEMALE CARRIERS

The special property of inactivation of one of the two X chromosome in females has been exploited for identification of carriers of X-linked immunodeficiency diseases (Table 57.1). A unique feature of the sex chromosomes is that males have one X and one Y, while females have two X chromosomes. As first hypothesized by Mary Lyon (1955), the problem of expression of double doses of genes from the female's X chromosomes is solved in mammals by inactivation of one X chromosome in all somatic tissues. A random inactivation of either the maternally or the paternally derived X chromosome occurs before differentiation of specialized tissues in the early female embryo (but, interestingly, not in the placenta, where the paternal X is preferentially inactivated). The random X inactivation imprint of each embryonic cell is maintained throughout subsequent cellular proliferation and differentiation, so that the contribution of maternal and paternal active X chromosomes in all tissues statistically follows a normal distribution. Thus the blood cells of normal women are chimeric for maternally and paternally derived active X chromosomes.

The ratio is usually around 50/50 but can vary substantially (Puck et al., 1992).

Carriers of certain X-linked immunodeficiencies have negative selection superimposed on the underlying inactivation pattern in the hematopoietic cell lineages affected by the gene defect they carry. If one of the X chromosomes bears a mutation in a gene that confers an intrinsic survival advantage upon cells of a particular lineage, then cells of that lineage will have skewed X inactivation. As indicated in Table 57.1, this is the case for B cells of carriers of XLA (Allen et al., 1994; Fearon et al., 1987); T, B, and NK cells of carriers of X-linked SCID (Conley et al., 1992; Puck et al., 1987; Wengler et al., 1993), and all hematopoietic cells of carriers of Wiskott-Aldrich syndrome (Prchal et al., 1980; Puck et al., 1990a). In contrast, there is no selective pressure on X inactivation in female carriers of X-linked CGD. Thus, as discussed above under immunological assays for CGD, two populations of granulocytes can be detected in female carriers, one with the mutated and one with the normal copy of the disease gene *CYBB*. In the unusual circumstance of extreme, constitutional unbalanced X inactivation, a female carrier of a *CYBB* mutation whose normal X chromosome is predominantly inactive can be clinically affected with CGD (Anderson-Cohen et al., 2003).

Assays of X inactivation have been used to identify female carriers in pedigrees for the purpose of gene mapping and positional cloning, first for X-linked SCID (Puck et al., 1987, 1990b) and recently for the *MAGT1* gene encoding a magnesium transporter defect that leads to T-cell impairment, severe Epstein-Barr virus infections, and lymphoma (Li et al., 2011) (Table 57.1). Methods to assay X inactivation based on methylation of the inactive X chromosome have been developed (Allen et al., 1994). However, X-inactivation analysis is an indirect test that can be misleading in women with constitutional unbalanced Lyonization, and it is no longer recommended for clinical carrier testing because specific mutation detection by DNA sequencing is more accurate.

MUTATION DETECTION

Several mutation detection techniques have been developed based on PCR amplification of genomic DNA or cDNA of individuals affected with immunodeficiencies or at-risk relatives, including single-strand conformation polymorphism (SSCP) (Hyashi et al. 1993; Orita et al., 1989; Puck et al., 1997a; Sheffield et al., 1993) and dideoxy fingerprinting (ddF) (Puck et al., 1997a; Sarkar et al., 1992). In these methods amplified DNA segments or dideoxy sequencing reactions, respectively, of up to 300 nucleotides in length are denatured by heating and then quickly chilled so that they assume a single-stranded conformation depending on their primary nucleotide sequence. The single-stranded segments are separated on nondenaturing acrylamide gels to preserve the secondary structure. Even single base changes alter the mobility of one or both of the single strands, as in Figure 57.3. In a study of 87 unrelated patients with X-linked (XSCID),

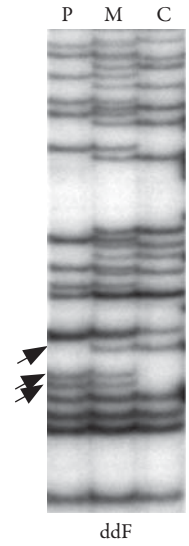

Figure 57.3 Dideoxy fingerprinting (ddF). Dideoxy T sequencing reactions of exon 5 of *IL2RG* in DNA from an XSCID patient, P; his carrier mother, M; and an unrelated control, C. Multiple bands with altered mobility are seen in the patient's lane, starting with two extra bands and a missing band marked by arrows. The band pattern in the lane of the heterozygous carrier mother is a composite of the patient's hemizygous mutated pattern and the control pattern.

IL2RG mutations were detected in all cases by ddF (Puck et al., 1997b).

However, determination of the nucleotide sequence of mutant DNA is now the gold standard for mutation detection as the costs of sequencing have fallen and fluorescence-based methods have become routine. Nucleotide changes detected by any DNA-based evaluation must be interpreted in light of expression studies and functional data before concluding that they are pathogenic mutations. Figure 57.4 shows detection by fluorescent sequencing of a two-base deletion in *IL2RG*. The wild-type or reference sequence and reading frame (upper tracing) and frame-shifted, deleted sequence of a patient with XSCID (lower tracing) can be observed to be superimposed in the central tracing from the patient's heterozygous mother. Sequence analysis of another XSCID kindred, shown in Figure 57.4, reveals the origin of the premature termination mutation in exon 3, V105X. The tracing of blood-derived DNA from the grandmother, whose son died of infections in infancy, shows only a minor admixture of an A base at the position that is a T in the wild type, indicating somatic mosaicism for this mutation in blood cells. The grandmother must also have had the new mutation in some of her egg cells, accounting for her affected son (not shown) and daughter, whose tracing in Figure 57.4 with equal amounts of mutant T and wild-type A is diagnostic of a heterozygous carrier state. Amniocyte DNA from the male fetus of this carrier demonstrates the presence of the mutant allele, in which a TAT tyrosine codon has become an immediate TAA termination codon. This fetus was therefore predicted to be affected with XSCID.

Detection of specific gene mutations has in some cases been scaled up and further automated (Lebet et al., 2007).

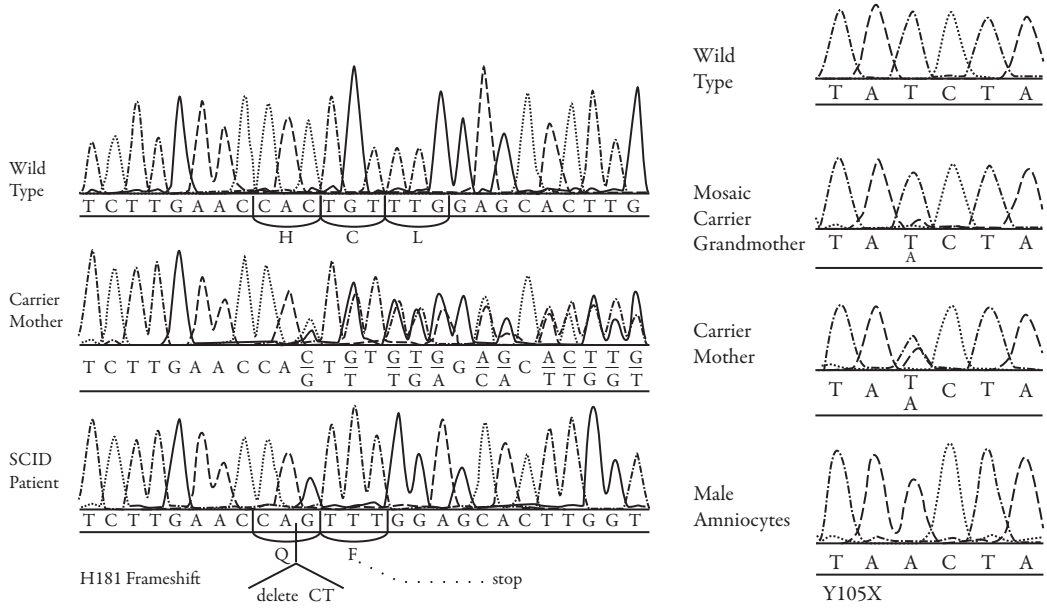

Figure 57.4 Fluorescence-based sequence determination. Right, wild type (upper), carrier (middle), and XSCID patient (lower) sequences from exon 3 of the *IL2RG* gene. The patient's deletion of two nucleotides in codon 181, normally specifying histidine (H), results in a frameshift specifying glutamine (Q) followed several further missense codons and a premature termination. The mother's genomic sequence is a composite of wild type and mutant, reflecting the two *IL2RG* alleles on her two X chromosomes. Left, *IL2RG* gene sequence in exon 3 showing wild type and three generations of a kindred with XSCID. The second tracing shows minor mosaicism in the grandmother for the mutant TAA allele, encoding a termination signal instead of the wild-type tyrosine (Y) residue. Her daughter, the mother of the patient, is a carrier of this mutation with roughly equal wild-type and mutant signals, while her male fetus is hemizygous for the mutant allele.

Detection of mutations in any of several SCID genes has used hybridization of PCR-enriched DNA to chips containing microarrays of partially overlapping oligonucleotides that span the gene's normal coding sequence.

HIGH-THROUGHPUT NEXT-GENERATION SEQUENCING

New massively parallel methods, referred to as next-generation sequencing (NGS), are now available to perform sequencing of several genes at once. Genomic DNA is sheared and fragments corresponding to the desired gene exons and surrounding nucleotides are captured by hybridization to beads or arrays of "bait" oligonucleotides. The captured DNA undergoes amplification and sequence determination, for example with bidirectional reads from the ends of each fragment, and sequence data are compiled by matching to the reference genome sequence using software programs. Arrays to capture DNA corresponding to many candidate disease genes are being used as an alternative to sequencing genes one at a time. On a somewhat larger scale, whole exome capture and sequencing, targeting about 2 percent of the human genome containing coding regions of genes, has become a very important tool for mutation detection in primary immunodeficiency diseases, particularly when the phenotype under study has been found in a population with consanguinity so that homozygous recessive mutations can be selected. While methods to capture, sequence, and analyze variants in DNA are still evolving, this technology has already led to many disease gene discoveries. Examples include defects in the signal transduction and activator of transcription protein STAT1 in chronic mucocutaneous candidiasis with impairment of IL-17 T cells (Liu et al., 2011); the Fas-associated death domain protein FADD in dominant immunodeficiency, immune dysregulation and cardiac disease (Bolze et al., 2010); and the p85α subunit of PI3 kinase in B-cell deficiency with agammaglobulinemia and colitis (Conley et al., 2012). In addition, known primary immunodeficiency genes have been associated with previously unappreciated phenotypes by whole exome sequencing, such as in discovery of susceptibility to papillomavirus-associated epidermodysplasia verruciformis caused by defects in the MST1 gene, already associated with defective T-cell production and survival (Crequer et al., 2021); Kaposi sarcoma in STIM1 deficiency (Byun et al., 2010); and ataxia-telangiectasia as a cause of T lymphocytopenia in otherwise healthy infants identified by newborn screening (see below) (Mallott et al., 2012).

The ultimate NGS for primary immunodeficiencies is whole genome sequencing, covering 50-fold more DNA than exome sequencing. Whole genome sequencing is not subject to bias introduced by capture methods, above, and is anticipated to become more widespread as methodology improves, costs decrease, and algorithms for analysis of the vast amounts of data improve. There are undoubtedly genomic loci that lie outside of exons and their immediate neighboring nucleotides but that control expression of genes. However, our abilities are still primitive when it comes to finding these amid the high level of variation that occurs outside of gene-coding regions.

GENETIC COUNSELING

Genetic testing has received increasing public attention because of the rapidly growing number of tests that can be performed for gene mutations associated with a wide variety of conditions. Testing is becoming publicly available in some cases before research has established its true ability to predict significant risk for development of a disease, such as breast cancer (Struewing et al., 1997). Our society is in the process of adjusting to increasing genetic information, but many issues, such as population screening, testing of minor children, protection of confidentiality, and insurability of persons carrying genetic mutations, remain to be resolved (American Society of Human Genetics Board of Directors and The American College of Medical Genetics Board of Directors, 1995; Clarke et al., 1994; Rowley et al., 1997). Unlike most other forms of medical evaluation, molecular genetic testing has implications not just for the proband, but also for relatives who share the same DNA. For all of these reasons, genetic counseling should precede genetic testing. Counseling should preferably be done by a geneticist or genetic counselor rather than the medical team primarily involved with treating an affected child, who may be critically ill. A genetic diagnosis that becomes available while a child is in the hospital should be reviewed with the family at a later time when the information can be processed more fully, questions can be answered, and implications for each family member can be sorted out.

Molecular diagnostic testing is usually undertaken to establish a diagnosis in an affected individual. Parents of an affected child are likely to be concerned about the risk of recurrence in a future pregnancy. For autosomal recessive diseases it is assumed that the parents are both likely to be carriers and the recurrence rate is one in four for pregnancies of either sex. For X-linked diseases in which the mother of an affected child is demonstrated to be a carrier, there is a 50 percent chance that each subsequent male pregnancy will be affected and a 50 percent chance that each female pregnancy will carry the mutation but not be affected with immunodeficiency.

Risks for being affected with the disease in question or for having affected children of one's own can be calculated for other family members. For example, a sibling of a proband affected with an autosomal recessive disease may be a carrier, but unless his or her mate is also a carrier of a mutation in the same gene (very unlikely unless the members of the couple share ancestry), the risk of having affected offspring is exceedingly low. On the other hand, sisters of males with X-linked diseases are at 50 percent risk of being carriers, in which case their male offspring will be at risk of being affected with the disease.

Now that many males with X-linked immunodeficiency diseases have been successfully treated and are reaching adulthood, it is important to help them understand that their reproductive risks have not changed, even if the disease in their blood cells has been permanently treated, such as by bone marrow transplantation. Affected males will pass on their mutation-bearing X chromosome to all of their daughters, who will be carriers; however, none of their sons will be affected because they will inherit the Y chromosome and not the X chromosome from their father.

Prenatal testing is possible for a large number of immunodeficiencies, and the chapters in Part II of this volume outline specific considerations for prenatal evaluation of each disorder. However, bringing the findings and techniques of research laboratories into a clinical setting has lagged. Because of high costs and low numbers of DNA-based diagnostic tests for each immunodeficiency gene, many tests have not yet been offered by clinical laboratories certified by the Clinical Laboratory Improvement Amendments of 1988 (CIA) Act Regulations (Regulations for implementing the Clinical Laboratory Improvement Amendments of 1988, MMWR, 1992). Fetal cells can be sampled by chorionic villus biopsy as early as the ninth week of pregnancy, or amniotic fluid can be obtained some weeks later as a source of fetal DNA for molecular testing. Fetal blood sampling between 16 and 20 weeks of pregnancy has also been used to evaluate the number and function of leukocytes in pregnancies at risk for well-defined abnormalities.

Preimplantation diagnosis is another procedure for families in which the specific mutations for the pregnancy at risk are known. This was first performed successfully in humans in the setting of cystic fibrosis in which the parents were both carriers of the common ΔF508 mutation in the *CFTR* gene (Ao et al., 1996). In vitro fertilization was carried out with superovulated eggs and sperm from the parents, embryos were grown to the eight-cell stage, and a single cell from each embryo was removed and genotyped by PCR. Embryos with an unaffected genotype were reimplanted into the mother. Of 22 couples in one early study, 5 had singleton pregnancies carried to term with the birth of healthy, unaffected infants.

Only limited information is available to indicate how parents with an at-risk pregnancy weigh the difficult choices that confront them. When a fetus is diagnosed as affected, the pregnancy may be terminated, but neonatal and even prenatal treatment options are increasingly available. The parents' perceptions of the burden of the disease are undoubtedly distinct for each immunodeficiency and are colored by the particular experience each person has shared with his or her affected relatives. A series of families followed with pregnancies at risk for XSCID indicated that most couples wanted to have prenatal testing, but few affected pregnancies were terminated (Puck et al., 1997a). In SCID, the improving outlook over the past three decades of bone marrow transplantation therapy has given families hope. Couples anticipating delivery of affected infants took advantage of the information gained through counseling and prenatal testing to learn about options for treatments. Pediatric centers performing bone marrow transplantation for immunodeficiency were contacted and financial and care arrangements were made. In some cases, the prenatal sample was HLA typed to identify potential matched bone marrow donors, either among the parents' healthy children or in an unrelated donor registry. Rare families have undergone experimental in utero bone marrow transplant; this treatment may become success-

ful with a variety of immune disorders (Flake et al., 1996; Flake & Zanjani, 1997).

A further development is the harvesting of cord blood at delivery as a source of autologous hematopoietic stem cells in which the gene defect could be corrected by gene therapy. Retroviral gene transfer has proven effective for XSCID and ADA-deficient SCID and has been undertaken for Wiskott-Aldrich syndrome, X-linked CGD, and other conditions (Chapter 61 and individual chapters). The development of leukemic clonal expansions of transduced cells due to retroviral insertional mutagenesis in recipients of gene therapy for XSCID and other diseases has identified a major hurdle, retroviral insertional mutagenesis. This complication has delayed widespread adoption of gene therapy, but vectors with improved safety are already in clinical trials.

POPULATION-BASED NEWBORN SCREENING FOR IMMUNODEFICIENCIES

Primary immunodeficiencies are underdiagnosed and frequently diagnosed late because of their low incidence and variable presentation, and because of insufficient awareness on the part of physicians and the public (Chan et al., 2011; Lindegren et al., 2004; Meyers et al., 2002). Screening of newborns for treatable genetic conditions has been a successful public-health measure to facilitate diagnosis and prompt intervention. As discussed in Chapter 10 and recently reviewed (Buckley, 2012; Puck, 2012; Verbsky et al., 2012; Kwan et al., 2013), newborn screening for SCID has become a practical reality in many states in the United States and is spreading to other countries.

Criteria to establish whether newborn screening is indicated for a given disease include that the disease be serious, treatable, and not detectable by physical examination in the nursery. Also, early treatment must be available and offer a better outcome, and incidence and test characteristics (cost, sensitivity, specificity) must make screening cost-effective (Wilson & Jungner, 1968). Several inherited immunodeficiencies meet several of these criteria, but to date only SCID has a screening test that has proven clinical utility (Chan & Puck, 2005; Routes et al., 2009; Kwan et al., 2013). The test, an assay of T-cell–receptor excision circles (TRECs) by PCR of DNA isolated from dried blood spots, reliably detects SCID. TRECs are formed as byproducts of T cell receptor rearrangement in the thymus, and a single PCR reaction across the joining point of the TREC is an indicator of thymic production of new, naïve T cells (Douek et al., 1998). TRECs are an excellent biomarker for newly produced naïve T cells. Low or absent T cells are characteristic of other conditions as well. Table 57.2 lists disease categories of T-cell lymphocytopenia detectable by TREC testing. The primary public health targets of TREC screening (Category I) are disorders that are life-threatening without prompt, definitive treatment by immune system restoring therapy: hematopoietic cell transplantation (Chapter 60), gene therapy (Chapter 61), enzyme therapy for ADA deficiency (Chapter 14), or thymus transplantation for complete DiGeorge syndrome (Chapter 45; Markert et al., 2009). Various genotypes of SCID and leaky SCID, discussed in Chapters 9 through 15, must be newly defined by minimal laboratory criteria (Table 57.1), because family history is usually negative and infectious complications will generally not have occurred at the time of diagnosis.

The remaining categories of T lymphocytopenia detected by TREC screening are considered secondary targets for which positive interventions can be made, such as avoiding live vaccinations, limiting exposure to infections, and in some instances administering immunoglobulin therapy or prophylactic antibiotics (Chapter 59). As experience is gained, unbiased data on the incidence, spectrum, and outcomes of infants with T lymphocytopenia detected by TREC screening are becoming available for the first time. In California, a state with a very diverse population and the largest number of births per year in the U.S., 993,724 newborns were screened in the first 2 years (Kwan et al., 2013). Of these, 50 (1/19,900; 0.005%) had significant T cell lymphocytopenia, as categorized in Table 57.2. Fifteen (1/66,250) required hematopoietic cell or thymus transplantation or gene therapy, including 11 infants with typical SCID, 3 with leaky SCID or Omenn syndrome, and 1 with complete DiGeorge syndrome. Survival to date in this group is 93%, higher than any published series of non-screened SCID patients. Other infants with low T cell numbers identified by screening included 6 with variant SCID or combined immunodeficiency, 12 with genetic syndromes associated with variable T cell impairment, 9 with secondary T lymphocytopenia, and 8 with preterm birth. All T lymphocytopenic infants avoided live vaccines and received appropriate interventions to prevent infections. The TREC test specificity was excellent, with only 0.08% of infants requiring a second test and 0.016% requiring lymphocyte phenotyping by flow cytometry.

Although low T-cell number is a common feature of many primary immunodeficiency disorders, the TREC test fails to identify several serious disorders characterized by impairment of T-cell function after the VDJ recombination step in T cell development that generates TRECs. Examples of non-detected disorders include ZAP70 deficiency, MHC class II deficiency, NEMO deficiency, and CD40 ligand deficiency, in which TRECs and T cells are present, but T cells are functionally compromised. In addition, antibody or neutrophil deficiencies, such as agammaglobulinemia or CGD, will not be detected. Identification of these disorders by newborn screening would be beneficial, and indeed B-cell immunoglobulin kappa chain gene recombination products can be assayed by DNA PCR in a manner similar to TRECs (Borte et al., 2012; Nakagawa et al., 2011). Even if additional tests are developed, newborn screening is unlikely to expand to diagnose all primary immunodeficiencies in a timely manner, even if whole exome or whole genome sequencing becomes routine. Thus, it is important for physicians to maintain awareness of the clinical presentations of immunodeficiency and to be vigilant for risk factors, including family history of early deaths, poor growth, recurrent or severe infections, or physical features, and to investigate accordingly.

Table 57.2 **CATEGORIES OF T LYMPHOCYTOPENIA DETECTABLE BY NEWBORN SCREENING WITH TRECS**

I. Conditions requiring immune system restoring therapy to establish T-lymphocyte production and maturation from hematopoietic stem cells

A. Typical SCID

T^- B^+ NK^+ or NK^- SCID with defects in IL-2 receptor common γ chain, IL-7 receptor α chain, JAK3 signaling kinase

T^- B^- NK^+ SCID with RAG1/2 or Artemis defects

T^- B^- NK^- SCID with ADA deficiency

Any other SCID genotypes that produce the phenotype of both <300 autologous T cells/uL and insufficient T-cell function revealed by maternal T-cell engraftment and/or PHA proliferation <10% of normal

B. Leaky SCID or Omenn syndrome, **due to incomplete (hypomorphic) defects in genes that when completely knocked out cause typical SCID**

300–1,500 T cells/uL, or higher numbers of autologous T cells that are oligoclonal and/or display memory phenotype (such as by expression of CD45RO)

No maternally engrafted cells

Defective lymphocyte function and/or lymphocyte dysregulation

C. "Complete" DiGeorge syndrome with profound thymic insufficiency, requiring definitive therapy by thymus transplantation

Absent production of autologous naïve T cells, not improving over several months of life

Usually associated with chromosome 22q11 deletion

Accompanied by other congenital anomalies (see Chapter 45)

II. Variant SCID or CID, **requiring immunological monitoring and possibly definitive transplantation therapy**

No identified gene defect

No maternal engraftment

300–1,500 T cells/uL, or higher numbers of autologous T cells that are oligoclonal and/or display memory phenotype (such as by expression of CD45RO)

Often, defective lymphocyte function or increased lymphocyte apoptosis

III. Syndromes with variably affected cellular immunity (that may be severe)

Partial DiGeorge syndrome with low T lymphocytes

CHARGE syndrome

Jacobsen syndrome

Trisomy 21

Ataxia-telangiectasia

DOCK8-deficient hyper-IgE syndrome

Cartilage-hair hypoplasia

RAC2 dominant interfering mutation

Additional rare multisystem genetic syndromes

IV. Secondary T lymphocytopenia

Neonatal cardiac surgery, with or without thymectomy

Neonatal leukemia

Gastroschisis

Third-spacing

Possibly severe prenatal HIV disease (hypothesized, but not observed to date)

V. Preterm birth alone

Generally resolves over time

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58.

IMMUNODEFICIENCY INFORMATION RESOURCES

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Lots of information is available from the Internet for more than 200 primary immunodeficiencies (PIDs), but it is scattered and often difficult to find. As the Internet is the primary source for most people looking for medical information, a navigation starting point and knowledge service are very much needed. We have compiled all the major data on immunodeficiencies into the ImmunoDeficiency Resource (IDR), a service that contains thousands of useful and interesting links in addition to our own pages. There are also many other kinds of resources available (e.g., for the diagnosis of PIDs) as well as patient and variation registries.

GENERAL PID INFORMATION

Some general PID information resources have been developed in recent years (Table 58.1). IDR and INFO4PI focus on PIDs and are tailored for different types of user groups, ranging from patients and their families to researchers and healthcare professionals. Other services, such as ORPHANET, are focused on rare diseases in general and include some PIDs.

IMMUNODEFICIENCY RESOURCE (IDR)

The IDR is a knowledge base whose goal is to provide a comprehensive integration of relevant clinical, biochemical, genetic, genomic, proteomic, structural, and computational data for PIDs (Samarghitean et al., 2007). The IDR provides structured and systematic information about PIDs and enables doctors, researchers, students, nurses, and patients to find validated information (Fig. 58.1A and B).

The IDR knowledge base was first released in 1999 (Väliaho et al., 2000, 2002, 2005). Currently, it contains information for 167 PIDs, including clinical as well as molecular data. IDR provides the most complete and up-to-date dataset available (Fig. 58.1A) and has been integrated with several internal and external databases and services. The contents of the IDR are validated and selected for different types of users. The search engine allows either a detailed or a broad search from a simple user interface. Also, multiple strings can be searched using Boolean logic. A PubMed search is possible directly by disease or gene name.

The service contains several introductory texts and has classification and diagnostic criteria, protocols, and guidelines for PIDs (Bonilla et al., 2005; Conley et al., 1999; de Vries & Clinical Working Party of the European Society for Immunodeficiencies [ESID], 2006). Recently, we have made a new classification for all PIDs based on data from the IDR and elsewhere by utilizing clustering and network methods (Samarghitean et al., 2009). PIDs were grouped based on clinical, pathological, and laboratory characteristics. Consensus of at least five methods provided a statistically supported novel classification that revealed previously unknown features and relationships of PIDs. Many independent properties of the diseases and related proteins had a homogeneous distribution when the severity and therapy of the diseases, functional classification of the proteins, and protein interaction network properties were analysed (Samarghitean et al., 2009).

The IDR has plenty of genome- and gene-related information available, such as genes and their loci, reference sequences, and markers. Links to genomic DNA and amino acid sequences, as well as three-dimensional structures of proteins, are available.

The IDR also contains instructional resources and analysis tools as well as advanced search routines. It has extensive cross-referencing and links to other services. The IDR pages are extensively hyperlinked to an integrated immunology glossary that contains descriptions for over 1,000 immunological terms.

The IDR lists collections of immunology-related data sources, including lectures on immunology and immunodeficiencies, animal models, and knockouts of immunodeficiency-related genes. Immunology and immunodeficiency societies, nursing societies, and patient organizations, as well as a number of patient pages, are listed.

Fact files, which form the core of the IDR, integrate biomedical knowledge from several heterogeneous sources and include information for disorders, genes, variations, protein sequences, online resources, organizations, and associations (Fig. 58.1C). Inherited Disease Markup Language (IDML), developed for the fact files, provides a standard method for exchanging genetic and clinical data, general disease descriptions, diagnostic information, and links to other related resources (Väliaho et al., 2005). The fact files make use of the following specifications, standards, and databases: HGNC gene nomenclature (Seal et al., 2011), UniProt (UniProt Consortium, 2012), GeneCard (Rebhan et al., 1998), and SOURCE (Diehn et al., 2003).

Although the IDR contains a huge amount of data, its logical organization and user-friendly interface allow easy access even to complex information. The IDR is continuously updated.

OTHER GENERAL KNOWLEDGE SERVICES

INFO4PI is a general PID information resource designed for diverse user groups. It is the official webpage of the Jeffrey Modell Foundation and contains useful information for doctors and patients and the lists of 10 warning signs for PIDs and four stages for immunological testing adopted in many countries.

ORPHANET provides information on rare diseases to health-care professionals, patients, and their relatives to contribute to the diagnosis, care, and treatment of these diseases. ORPHANET includes an encyclopaedia for professionals, with expert-authored and peer-reviewed information for rare, mostly genetic, diseases and an Encyclopedia for patients. ORPHANET includes information on clinics, clinical laboratories, research activities, and patient organizations. PID-related information is cross-linked within IDR fact files. ORPHANET provides validated and up-to-date information about rare diseases in different languages and also contains information about initiatives of the EU Commission on Rare Diseases Task Force (RDTF, http://www.rdtf.org) and International Rare Diseases Research Consortium (IRDiRC, http://www.irdirc.org/).

A list of other useful websites, which contains information about PIDs, is provided in Table 58.1.

DIAGNOSIS TOOLS

Internet-distributed diagnosis tools for PIDs can be grouped between databases, which archive gene and clinical test laboratories; medical expert systems, which use different reasoning mechanisms to aid in diagnosis; and other tools helpful for diagnostic procedures.

IMMUNODEFICIENCY DIAGNOSTIC REGISTRY (IDDIAGNOSTICS)

The diagnosis of immunodeficiencies can be difficult because symptoms may be similar in several disorders. As many PIDs are rare, often there is no statistical information about signs and symptoms available. Early and reliable diagnosis is often crucial for efficient treatment of these diseases since delayed diagnosis and management of PIDs can lead to severe and irreversible complications, even resulting in the death of the patient.

The definitive diagnosis of PIDs in many cases depends both on genetic and clinical tests, since the physical signs may be nonspecific, very discreet, or absent. Due to the rareness of PIDs there are generally not many laboratories analyzing a particular disease. IDdiagnostics contains one registry for laboratories performing genetic tests and one registry for clinical testing for patients with a suspected PID (Samarghitean et al., 2004).

In IDdiagnostics, contact addresses for laboratories performing genetic diagnoses are provided, along with information about the assay method(s) used. There are also details on the turnaround time, how often the samples are run, and how many samples are studied annually. Laboratories can have special requirements for samples and their handling, data for which are also available from the IDdiagnostics service. Physicians planning to send samples should first consult the laboratories to learn about conditions, shipping details, and the expected time for the test and price (if any).

The IDdiagnostics database has multiple search facilities. Further information and submission pages (Fig. 58.1D) for both genetic and clinical testing can be found at http://bioinf.uta.fi/IDdiagnostics/. Laboratories carrying out testing are asked to provide their information, as inclusion in the registries is on a voluntary basis.

OTHER DIAGNOSTIC INFORMATION RESOURCES

The GeneTests service includes an international directory of genetic testing laboratories and genetics and prenatal diagnosis clinics. GeneTests will probably be continued at University of Washington since summer 2013. GeneReviews are expert-authored, peer-reviewed disease descriptions. IDR fact files and IDdiagnostics are linked to PID-related information in GeneTest.

The European Directory of DNA Diagnostic Laboratories (EDDNAL) disseminates information about DNA-based diagnostic services for rare genetic conditions in Europe. Information about PID gene test laboratories from EDDNAL is cross-linked in IDR fact files and in IDdiagnostics.

Some diagnostic protocols have been implemented on the Internet. The multistage diagnostic protocol designed for nonimmunologists (de Vries & Clinical Working Party of the European Society for Immunodeficiencies [ESID], 2006) has been reinterpreted as linked web pages on the UKPIN site. However, there is a new version of the protocol published (deVries & European Society for Immunodeficiencies

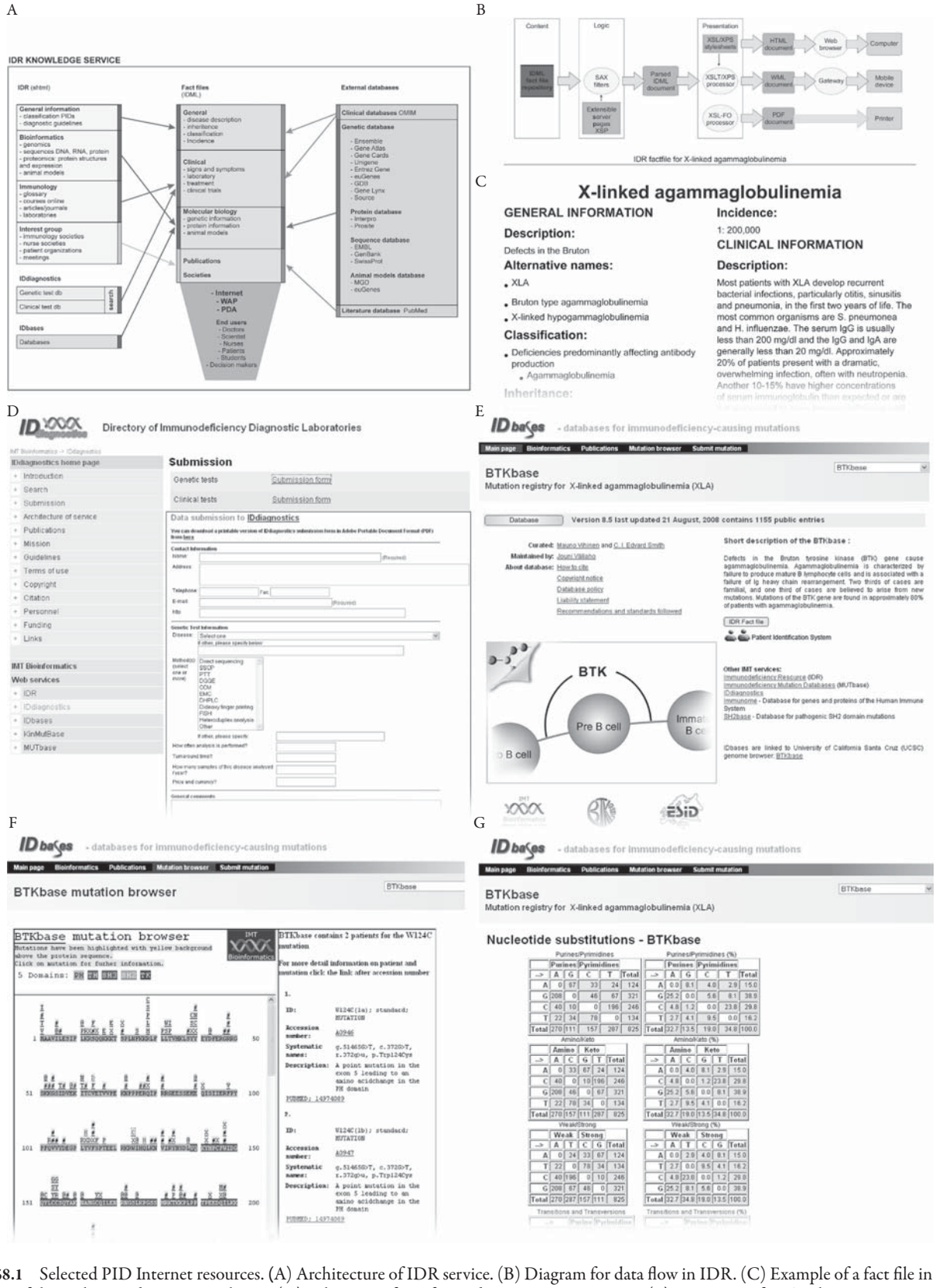

Figure 58.1 Selected PID Internet resources. (A) Architecture of IDR service. (B) Diagram for data flow in IDR. (C) Example of a fact file in IDR; only part of the multipage document is shown. (D) Submission form for IDdiagnostics genetic tests. (E) Home page for BTKbase, a mutation registry for X-linked agammaglobulinemia caused by Bruton tyrosine kinase mutations. (F) A BTKbase, showing distribution of mutations within protein domains. (G) IDbases contain information for mutation statistics on DNA, RNA, and protein level.

Table 58.1 INTERNET SITES RELATED TO PIDS

ABBREVIATION	URL	GROUP OR SOCIETY NAME	REFERENCES
ASCIA	http://www.immunodeficiency.org.au/	ASCIA Primary Immunodeficiency Diseases Register of Australia and New Zealand	Kirkpatrick and Riminton, 2007
ESID	http://www.esid.org	European Society for Immunodeficiencies	Guzman et al., 2007, Eades-Perner et al., 2007
IDF	http://primaryimmune.org	Immune Deficiency Foundation (USA)	
INGID	http://www.ingid.org	International Nursing Group for Immunodeficiencies	
IPIDR	http://ipidr.tums.ac.ir/	Iranian Primary Immunodeficiencies Registry	Rezaei et al., 2006, Aghamohammadi et al., 2002, 2006
IPINET	http://www.aieop.org	Italian Primary Immunodeficiencies Network	Plebani et al., 2004, Quinti et al., 2007
IPOPI	http://www.ipopi.org	International Patient Organisation of Primary Immunodeficiencies	
NPI	http://www.info4pi.org	National Primary Immunodeficiencies Resource Center (USA)	
LAGID	http://www.lagid.lsuhsc.edu/	Latin American Group for Immunodeficiencies	
RAPID	http://rapid.rcai.riken.jp	Resource of Asian Primary Immunodeficiency Diseases	Keerthikumar et al., 2009
UKPIN	http://www.ukpin.org.uk	UK Primary Immunodeficiency Network	
USIDNET	http://www.usidnet.org	U.S. Immunodeficiency Network	

Members, 2011). Recommendations for diagnosis and treatment of some PIDs, both in English and in Italian, can be found on IPINET, the Italian Primary Immunodeficiency Network. CEREDIH, the French National Immunodeficiency Center, distributes information about French centers and laboratories performing PID diagnosis. The 10 warning signs list for children and 12 warning signs for adults are also available. There are also articles and a diagnostic protocol. This service is written only in French.

PIDEXPERT

Medical expert systems (MESs) are computer software systems that use a set of rules applied to knowledge extracted from human experts and generated by computational analyses. MESs can help in diagnosis and report generation, improve consistency in decisions, and increase timeliness in decision-making and productivity (Samarghitean & Vihinen, 2008). MESs produce patient-specific and situation-specific recommendations. MESs can be integrated with other applications, such as electronic patient records, systems for prescribing and dispensing medicines, and other information systems used in health-care settings.

PIDexpert is an MES to help with the diagnosis and management of PIDs (Samarghitean et al., in preparation). PIDexpert generates a differential diagnosis from clinical symptoms and suggests any potentially useful further clinical and laboratory information required. The main components of the shell are a knowledge base, a query base, an inference engine, and a graphical user interface. The query base and knowledge base include data and facts from the IDR, IDdiagnostics, IDbases (Piirilä et al., 2006), ESID registry (Guzman et al., 2007), from different national registries such as IPINET, CEREDIH, UKPIN, USIDnet, and from the literature (Ochs et al., 2007; Rezaei et al., 2008; Spickett, 2006; Stiehm et al., 2004). The data are mainly informal and heuristic. The ESID/PAGID diagnostic guidelines (Conley et al., 1999) and practice parameters for the diagnosis and management of PIDs established by the American Academy of Allergy, Asthma and Immunology (AAAAI), the American College of Allergy, Asthma and Immunology (ACAAI), and the Joint Council of Allergy, Asthma and Immunology (JCAAI) (Bonilla et al., 2005) were coded into the system. These guidelines provide heuristics for possible/probable/definitive diagnosis for some of the most common PIDs.

The inference engine searches for best-fitting patterns instead of the nearest neighbor. The system also identifies other conditions that might be associated with the disorder and suggests how the diagnosis could be confirmed.

PATIENT REGISTRIES

Several PID patient registries have been developed in different countries during the past two decades. The information in them can help in making a diagnosis for a patient.

ESID has collected information about immunodeficiency patients into a large European registry (Guzman et al., 2007). In April 2013, the database counted16,547 patient datasets from 96 centers. There are individual databases for each PID with a common core dataset containing information about diagnosis, therapy, quality of life, and important laboratory data. There are also disease-specific data models for the most prevalent PIDs. A dedicated tool allows submission of variation data to the IDbases. Access to the data is carefully controlled and the resource is protected behind secure firewalls.

The United States Immunodeficiency Network (USIDnet) runs an identical registry for U.S. patients. Results from the national registry of U.S. residents with X-linked agammaglobulinemia provided an updated clinical view of the disorder in a large cohort of patients (Winkelstein et al., 2006).

A resource of Asian Primary Immunodeficiency Diseases (RAPID) hosts information on sequence variations and expression at the mRNA and protein levels for all PIDs. There is also information about protein–protein interactions, mouse studies, and gene-expression profiles in various organs and cell types. RAPID also has a tool to visualize mutations on PID protein 3D structures (Keerthikumar et al., 2009).

The Latin American Group for Primary Immunodeficiency Diseases (LAGID) collects epidemiological information about PIDs in Latin America using a uniform questionnaire and a computerized database (Leiva et al., 2007; Zelazko et al., 1998). The ESID online system has been adopted by LAGID. ASCIA PID Register of Australia and New Zealand has already been used to describe the prevalence of PIDs in Australia and the need for specific therapies (Baumgart et al., 1997).

There are also a number of other national PID registries that, like the ones described above, are not publicly accessible. Italian IPINET (Luzi et al., 1983, 1991; Plebani et al., 2002, 2004; Soresina et al., 2008) collects patient information including the pedigree, date of diagnosis, immunological data and clinical manifestations, laboratory data, and information about replacement therapy. The Spanish Registry for Primary Immunodeficiencies (REDIP) contains demographic information, data about diagnosis, age at diagnosis, family history, and treatment (Matamoros Flori et al., 1997). The Iranian PID Registry has followed 930 patients over a period of 30 years using a four-page questionnaire. The registry includes personal and demographic data, family history, clinical manifestations, laboratory findings, treatment, and follow-up data. The diagnosis of patients has been confirmed by the scientific committee of the registry using standard criteria (Rezaei et al., 2006).

IMMUNODEFICIENCY VARIATION DATABASES (IDBASES)

Information about PID-related genetic variations has been collected in the ImmunoDeficiency variation databases (IDbases) since the mid-1990s. Currently, 131 freely available IDbases are available (Table 58.2). Scientists analyzing variations are requested to submit their data either by contacting the curators or by using the Web submission available on the homepages of the databases. Many of the IDbases contain plenty of information in addition to details of the actual variation. The variation entries are linked to other data sources, such as sequence databanks (Benson et al., 2013), PubMed, OMIM (http://omim.org/), and UniProt (UniProt Consortium, 2009). The other standards and recommendations followed include the HUGO Gene Nomenclature Committee (HGNC) gene names (Seal et al., 2011) and Human Genome Variation Society (HGVS) recommendations for mutation nomenclature (den Dunnen & Antonarakis, 2000), database content, and submission for recommendations.

The MUTbase program suite (Riikonen & Vihinen, 1999) provides interactive and quality-controlled submission of information to IDbases and generates the pages for Internet distribution (Fig. 58.1E). There are several web pages to show the types of variations and their distribution within protein domains as well as in the exons and introns of the gene (Fig. 58.1F). The user can click the interactive pages to obtain more information. Interactive graphic pages show the number of disorder-related families having different variation types at each residue.

IDbases contains variation information for 9.950 patients from 8,623 families having a total of 3,627 different variation events. Figure 58.2 shows the distribution of PID

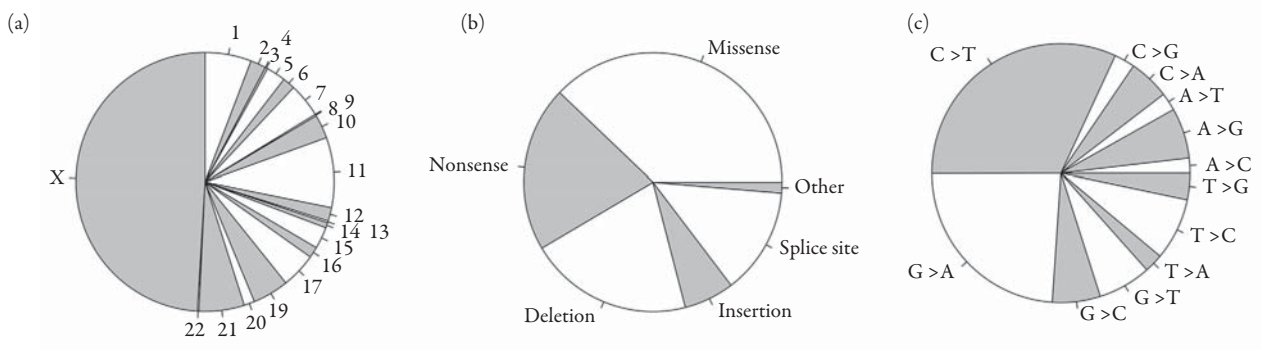

Figure 58.2 Distribution of variations in IDbases summed over all the registries at the Bioinformatics group. (A) Distribution of variations in chromosomes. (B) Distribution of variation types. (C) Distribution of nucleotide changes in PID-causing variations.

Table 58.2 IDBASES FOR PID VARIATIONS.

DATABASE	IMMUNODEFICIENCY	PUBLIC CASES	REFERENCE
ADAbase	Adenosine deaminase deficiency	74	
AICDAbase	Non–X-linked hyper-IgM syndrome	79	
AIREbase	Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECEC)	275	
AK2base	Reticular dysgenesis	11	
AP3B1base	Hermansky-Pudlak syndrome 2	11	
BIRC4base	X-linked lymphoproliferative syndrome (XLP)	19	
BLMbase	Bloom syndrome	146	Rong et al., 2000
BLNKbase	BLNK deficiency	1	
BTKbase	X-linked agammaglobulinemia (XLA)	1252	Väliaho et al., 2006
C1QAbase	C1q α polypeptide deficiency	9	
C1QBbase	C1q β polypeptide deficiency	5	
C1QCbase	C1q γ polypeptide deficiency	5	
C1Sbase	C1s deficiency	7	
C2base	C2 deficiency	3	
C3base	C3 deficiency	13	
C5base	C5 deficiency	8	
C6base	C6 deficiency	13	
C7base	C7 deficiency	35	
C8Bbase	C8B deficiency	60	
C9base	C9 deficiency	9	
CARD9base	Chronic mucocutaneous candidiasis	7	
CASP10base	Autoimmune lymphoproliferative syndrome type II	2	
CASP8base	Caspase 8 deficiency	2	
CD19base	CD19 deficiency	5	
CD247base	CD3ζ deficiency	2	
CD3Dbase	Autosomal recessive CD3δ deficiency	7	
CD3Ebase	Autosomal recessive CD3ε deficiency	2	
CD3Gbase	Autosomal recessive CD3γ deficiency	4	
CD40Lbase	X-linked hyper-IgM syndrome (XHIM)	250	Notarangelo et al., 1996 Thusberg and Vihinen, 2007
CD40base	CD40 deficiency	4	
CD55base	Decay-accelerating factor (CD55) deficiency	9	
CD59base	CD59 deficiency	1	
CD79Abase	Igα deficiency	2	
CD79Bbase	Igβ deficiency	1	
CD8Abase	CD8 deficiency	4	
CEBPEbase	Neutrophil-specific granule deficiency	3	
CFDbase	Factor D deficiency	6	
CFHbase	Factor H deficiency	107	
CFIbase	Complement factor I deficiency	19	
CFPbase	Properdin deficiency	36	
CIITAbase	MHC II transactivating protein deficiency	8	
CTSCbase	Papillon-Lefevre syndrome	137	

Table 58.2 (CONTINUED)

DATABASE	IMMUNODEFICIENCY	PUBLIC CASES	REFERENCE
CXCR4base	WHIM syndrome	33	
CYBAbase	Autosomal recessive p22phox deficiency	86	
CYBBbase	X-linked chronic granulomatous disease (XCGD)	1387	Roos, 1996
DCLRE1Cbase	Artemis deficiency	55	
DKC1base	Hoyeraal-Hreidarsson syndrome	13	
DNMT3Bbase	ICF syndrome	20	Lappalainen and Vihinen, 2002
ELA2base	Cyclic neutropenia and severe congenital neutropenias	159	
FASLGbase	Autoimmune lymphoproliferative syndrome type 1B (ALPS1B)	2	
FCGR1Abase	CD64 deficiency	4	
FCGR3Abase	Natural killer cell deficiency	3	
FERMT3base	Leukocyte adhesion deficiency type III	25	
FOXN1base	T-cell immunodeficiency, congenital alopecia, and nail dystrophy	2	
FOXP3base	Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX)	49	
G6PC3base	Severe congenital neutropenia	22	
GFI1base	Severe congenital neutropenia and nonimmune chronic idiopathic neutropenia of adults	5	
HAX1base	Severe congenital neutropenia (Kostmann disease)	39	
ICOSbase	ICOS deficiency	11	
IFNGR1base	IFN-γ1-receptor deficiency	79	
IFNGR2base	IFN-γ2-receptor deficiency	9	
IGHG2base	IgG2 deficiency	5	
IGHMbase	μ heavy-chain deficiency	20	
IGLL1base	λ5 surrogate light-chain deficiency	1	
IKBKGbase	NEMO deficiency	85	
IL12Bbase	Interleukin-12 p40 deficiency	14	
IL12RB1base	Interleukin-12 receptor β1 deficiency	58	
IL2RAbase	IL2RA deficiency	2	
IL7Rbase	Interleukin-7 receptor α deficiency	7	
IRAK4base	IRAK4 deficiency	23	
ITGB2base	Leukocyte adhesion deficiency I (LAD I)	129	
JAK3base	Autosomal recessive severe combined JAK3 deficiency	32	Vihinen et al., 2000; Notarangelo et al., 2001
LIG1base	DNA ligase I deficiency	1	
LIG4base	LIG4 syndrome	12	
LRRC8Abase	Non-Bruton type autosomal dominant agammaglobulinemia	1	
LYSTbase	Chediak-Higashi syndrome	37	
MAPBPIPbase	Endosomal adaptor protein p14 deficiency	4	
MASP2base	MASP-2 deficiency	1	
MLPHbase	Griscelli syndrome type 3 (GS3)	1	
MPObase	Myeloperoxidase deficiency	39	
MRE11Abase	Ataxia-telangiectasia–like disorder (ATLD)	19	
MYO5Abase	Griscelli syndrome type 1 (GS1)	2	
NCF1base	Autosomal recessive p47phox deficiency	89	
NCF2base	Autosomal recessive p67phox deficiency	102	

(continued)

Table 58.2 (CONTINUED)

DATABASE	IMMUNODEFICIENCY	PUBLIC CASES	REFERENCE
NFKBIAbase	Autosomal dominant anhidrotic ectodermal dysplasia and T-cell immunodeficiency	2	
NHEJ1base	Combined immunodeficiency (CID) associated with microcephaly and increased cellular sensitivity to IR	12	
NPbase	PNP deficiency	16	
NRASbase	Autoimmune lymphoproliferative syndrome type IV	1	
ORAI1base	Severe combined immunodeficiency	4	
PRF1base	Familiar hemophagocytic lymphohistiocytosis type II (FHL2)	157	
PRKDCbase	Severe combined immunodeficiency (DNA-PKc)	1	
PTPRCbase	CD45 deficiency	9	
RAB27Abase	Griscelli syndrome type 2 (GS2)	51	
RAC2base	Neutrophil immunodeficiency syndrome	1	
RAD50base	Nijmegen breakage syndrome-like syndrome	1	
RAG1base	Autosomal recessive severe combined RAG1 deficiency	90	Sobacchi et al., 2006
RAG2base	Autosomal recessive severe combined RAG2 deficiency	44	Sobacchi et al., 2006
RASGRP2base	Leukocyte adhesion deficiency III	2	
RFX5base	MHCII promoter X box regulatory factor 5 deficiency	8	
RFXANKbase	Ankyrin repeat containing regulatory factor X-associated protein deficiency	30	
RFXAPbase	Regulatory factor X-associated protein deficiency	7	
RNF168base	Ataxia telangiectasia	1	
SBDSbase	Shwachman-Diamond syndrome	223	
SERPING1base	Hereditary angioedema	290	
SH2D1Abase	X-linked lymphoproliferative syndrome (XLP)	133	Lappalainen et al., 2008
SLC35C1base	Leukocyte adhesion deficiency II (LAD II)	9	
SMARCAL1base	Schimke immuno-osseous dysplasia	43	
SP110base	Hepatic veno-occlusive disease with immunodeficiency syndrome (VODI)	6	
SPINK5base	Netherton syndrome	87	
STAT1base	STAT1 deficiency	9	
STAT2base	STAT2 deficiency	5	
STAT3base	Hyper-IgE syndrome	94	
STAT5Bbase	Growth hormone insensitivity with immunodeficiency	4	
STIM1base	STIM1 deficiency	2	
STX11base	Familial hemophagocytic lymphohistiocytosis 4	19	
STXBP2base	Hemophagocytic lymphohistiocytosis	27	
TAP1base	TAP1 deficiency	6	
TAP2base	TAP2 deficiency	4	
TAPBPbase	Tapasin deficiency	1	
TAZbase	Barth syndrome	87	
TCN2base	Transcobalamin II deficiency	12	
TLR3base	Influenza-associated encephalopathy	3	
TMC6base	Epidermodysplasia verruciformis	11	
TMC8base	Epidermodysplasia verruciformis	11	
TNFRSF13Bbase	TACI deficiency	70	
TYK2base	TYK2 deficiency	1	
UNC13Dbase	Familial hemophagocytic lymphohistiocytosis 3	68	

Table 58.2 (CONTINUED)

DATABASE	IMMUNODEFICIENCY	PUBLIC CASES	REFERENCE
UNC93B1base	UNC93B deficiency (herpes simplex encephalitis)	2	
UNGbase	UNG deficiency (hyper-IgM syndrome type 5)	4	
WASbase	Wiskott-Aldrich syndrome (WAS)	264	
ZAP70base	Autosomal recessive severe combined ZAP70 deficiency	17	

All available at http://bioinf.uta.fi/IDbases.

If not otherwise indicated, reference is Piirilä et al., 2006.

causing mutations in chromosomes and the mutation types and nucleotide changes. C>T and G>A substitutions are overrepresented due to their appearance in mutation-prone CpG dinucleotides.

We collaborate with the RefSeqGene (http://www.ncbi.nlm.nih.gov/RefSeq/) and LRG projects (http://www.lrg-sequence.org) in replacing our IDRefSeqs with updated and stable reference sequences describing the genes in three levels: genomic, RNA, and protein sequence. These will provide a stable genomic framework for reporting variations with a permanent and core content that will never change. In IDbases, in addition to the disease-causing variations, polymorphisms or benign variations are also included in separate files. A lot of statistical data is provided for each gene about amino acid substitutions, codon changes, and nucleotide substitutions (Fig. 58.1G).

IDbases can provide new insights into both genotype–phenotype correlations in patients as well as protein structure–function relationships for the encoded proteins. The information may be essential in developing new treatments, including in drug design. The IDbases are linked to the University of California Santa Cruz (UCSC) genome browser (Giardine et al., 2007; Karolchik et al., 2008). The mutation data can be easily viewed with PhenCode (Giardine et al., 2007), along with other genetic and variation information. The IDbases are developed along with recommendations jointly produced with the GEN2PHEN consortium (http://www.gen2phen.org/).

Several PID mutation databases are maintained in other laboratories (Table 58.3). The coverage and depth of detail vary in these registries. Currently, there exists a locus-specific mutation database for almost all PIDs in which gene defects are known.

OTHER IMMUNODEFICIENCY-RELATED SERVICES

Gene defects are relatively easy to identify; however, the interpretation of the effects of sequence variations and elucidation of the detailed molecular mechanisms of genetic diseases are much more difficult. Amino acid substitutions may have diverse effects on protein structure and function (Thusberg & Vihinen, 2009). Numerous methods can be used for predicting the effects of amino acid substitutions and are collected in the Pathogenic-Or-Not Pipeline (PON-P) website, which is freely available at http://bioinf.uta.fi/PON-P.

A bioinformatics approach has been applied to predict novel PID candidate genes (Ortutay & Vihinen, 2009a). A total of 26 putative PID genes were prioritized. The method combines information about protein interaction network properties and Gene Ontology terms. The analysis was based on a dataset for the immunome—that is, the entirety of genes and proteins essential for mounting immune responses (Ortutay & Vihinen, 2006). The approach utilizes protein interaction network information available from the Immunome Knowledge Base (Ortutay & Vihinen, 2009b). The identified disease gene candidates are mainly involved in cellular signaling, including receptors, protein kinases and adaptors, and binding proteins as well as enzymes (Ortutay & Vihinen, 2009a).

CONCLUSIONS

Many bioinformatics tools and information resources such as IDR, IDbases, and IDdiagnostics are freely available on the Internet. These services are clearly needed, as shown, for example, by the user statistics for IDR for the year 2012: 145,207 downloads from 114 countries. Constantly updated PID information resources are valuable for everybody working with, suffering from, or interested in these disorders.

PID patient registries, due to their nature, are not publicly accessible. In the future we can expect electronic information resources and computational tools to further help health-care professionals in diagnosis, in keeping up with the deluge of new data, and by providing reliable information and analyses, diagnosis, and prediction tools.

ACKNOWLEDGMENTS

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Table 58.3 IDBASES MAINTAINED ELSEWHERE

GENE	DISEASE	URL	PUBLIC CASES	REFERENCES
AP3B1	Hermansky-Pudlak syndrome 2	http://albinismdb.med.umn.edu/hps2mut.htm	4	
			47	
CASP10	Autoimmune lymphoproliferative syndrome type II	http://www.niaid.nih.gov/topics/alps/Pages/default.aspx	2	
CD46	Haemolytic uremic syndrome (HUS)	http://www.fh-hus.org/	1	Saunders et al., 2007
CFH	Haemolytic uremic syndrome (HUS)	http://www.fh-hus.org/	78	Saunders et al., 2007
CFI	Haemolytic uremic syndrome (HUS)	http://www.fh-hus.org/	1	
CTSC	Papillon-Lefevre syndrome	http://www.genetics.pitt.edu/mutation/pls/	48	
FANCA	Fanconi anemia complementation group A	http://www.rockefeller.edu/fanconi/genes/jumpa	1,119	Levran et al., 2005
FANCC	Fanconi anemia complementation group B	http://www.rockefeller.edu/fanconi/genes/jumpb	19	
FANCC	Fanconi anemia complementation group C	http://www.rockefeller.edu/fanconi/genes/jumpc	286	
FANCD2	Fanconi anemia complementation group D2	http://www.rockefeller.edu/fanconi/genes/jumpd2	78	
FANCE	Fanconi anemia complementation group E	http://www.rockefeller.edu/fanconi/genes/jumpe	46	
FANCF	Fanconi anemia complementation group F	http://www.rockefeller.edu/fanconi/genes/jumpf	40	
FANCG	Fanconi anemia complementation group G	http://www.rockefeller.edu/fanconi/genes/jumpg	36	
FANCL	Fanconi anemia	http://www.rockefeller.edu/fanconi/genes/jumpl	4	
FAS	Autoimmune lymphoproliferative syndrome type Ia	http://research.nhgri.nih.gov/ALPS/alpsIa_mut.shtml	63	
IL2RG	X-linked SCID	http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=IL2RG	200	Puck et al., 1989
LPIN2	Majeed syndrome	http://fmf.igh.cnrs.fr/ISSAID/infevers/	8	
LYST	Chediak-Higashi syndrome	http://albinismdb.med.umn.edu/chs1mut.html	15	
MEFV	Familial Mediterranean fever	http://fmf.igh.cnrs.fr/ISSAID/infevers/	184	
MVK	Hyper-IgD syndrome and periodic fever	http://fmf.igh.cnrs.fr/ISSAID/infevers/	106	
NLRP3	Familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological cutaneous and articular syndrome	http://fmf.igh.cnrs.fr/ISSAID/infevers/	116	
NLRP7	Recurrent hydatidiform moles and reproductive wastage	http://fmf.igh.cnrs.fr/ISSAID/infevers/	142	
NOD2	Blau syndrome, Crohn's disease, early-onset sarcoidosis	http://fmf.igh.cnrs.fr/ISSAID/infevers/	104	
PSTPIP1	Pyogenic sterile arthritis, pyoderma gangrenosum, and acne syndrome	http://fmf.igh.cnrs.fr/infevers/	5	
SERPING1	Hereditary angioedema	http://hae.enzim.hu/	93	
TAZ	Barth syndrome	http://www.barthsyndrome.org/english/View.asp?x=1357	172	
TNFRSF1A	Tumor necrosis factor receptor-associated periodic syndrome	http://fmf.igh.cnrs.fr/ISSAID/infevers/	91	
WAS	Wiskott-Aldrich syndrome and X-linked thrombocytopenia	http://pidj.rcai.riken.jp/waspbase/	441	

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59.

CONVENTIONAL THERAPY OF PRIMARY IMMUNODEFICIENCY DISEASES

E. Richard Stiehm and Helen M. Chapel

Long-term care of patients with primary immunodeficiencies is aimed at preventing infections and their long-term sequelae as well as treating breakthrough infections. The extent of appropriate infection prevention, as well as treatment for the complications, depends on the nature of the immune deficiency. For this reason, this chapter is divided into two sections: the first describes general measures applicable to all forms of immune deficiency, and the second details specific measures for patients with the most common individual disorders.

The management of all forms of immunodeficiency diseases requires interaction between generalists and specialists with experience and expertise in primary immune deficiencies. This usually requires a team of a primary care pediatrician or internist and an immunologist, with assistance and advice from other specialists (e.g., dermatologists, pulmonologists, gastroenterologists, rheumatologists, and radiologists) as necessary, since it is important that patients continue to have the benefit of optimal diagnostic procedures and new drugs for all organ-based complications that they may develop.

For example, patients with bronchiectasis following recurrent pneumonia (Chapel et al., 2008) need not only regular monitoring with pulmonary function tests and computed tomography (CT) scans but also access to chest physiotherapists. Those with asthma require the latest and most effective bronchodilators. In the same way that the immune system is multiorgan, the failure of this system manifests diverse complications requiring organ-based specialist medical care.

PART I: GENERAL CONSIDERATIONS

PREVENTION

Prevention of primary immunodeficiency is limited and is applicable only to those situations with a previous affected infant or a positive family history. These considerations are of particular importance in countries where consanguineous marriages are common. Preventive measures include providing genetic counseling to a family at risk, arranging for prenatal diagnosis, and, in certain circumstances, preimplantation diagnosis or termination of pregnancy.

Prevention of infections is not always possible, but several precautions are offered in Table 59.1 to minimize exposure to contagious illness and environmental sources of infection wherever possible.

TREATMENT

Because many patients with immunodeficiency will have a normal lifespan on immunoglobulin (IG) (Chapel et al., 2008), stem cell transplants, or other therapies, they and their parents and relatives must avoid making the patient an emotional cripple through overprotection. A child should be encouraged to venture outdoors, play with other children in small groups, attend nursery and regular school, and participate in sports and other extracurricular activities that involve pulmonary exercise. The aim is to teach patients to live in a near-normal fashion with their disease, much like a diabetic patient on insulin therapy.

Adolescents, and even adults, need advice about the importance of regular exercise (especially to improve bronchiectasis). They should not smoke, inhale secondhand smoke, or use illegal drugs. Like tobacco, marijuana is a pulmonary irritant and should be avoided. Sniffing drugs can result in inhaled nasal bacteria and pneumonia.

School health officials should be notified of the child's illness, so that exposure to infectious disease is minimized and prompt attention to injuries is provided.

Patients with immunodeficiency require extraordinary amounts of care to maintain general health and nutrition, prevent emotional problems related to their illness, and manage

Table 59.1 GENERAL MEASURES TO PREVENT INFECTIONS IN IMMUNODEFICIENCY

- Take precautions recommended by the physician for the particular immune deficiency.
- Keep indicated vaccines updated as recommended by the pediatrician for the particular immune defect (see text).
- Avoid people with an obvious infection.
- Avoid raw, undercooked, unwashed, unpasteurized foods/liquids.
- Avoid construction/excavation sites, mulch, hay stacks, compost turning.
- Wear gloves when gardening.
- Avoid well water and lake water.
- Avoid high-risk sexual activity.
- Avoid tattoos and piercing.
- Avoid tobacco, marijuana, illegal drugs.
- Consider avoiding farms, petting zoos, fare animals, puppies, kittens, stray animals, and exotic pets if rare infections are a particular risk.
- Take sick animals to vets right away.
- Ask someone else to clean fish tanks, litter boxes, animal feces, or bird cages.
- Wash hands regularly, especially after pet contact.

their numerous infectious episodes. This regimen applies equally to adults and children. Usually, no special dietary limitations are necessary; the aim is to provide a well-rounded, nutritious diet.

Patients with immunodeficiency should be protected from unnecessary exposure to infection, particularly to those types of infection to which they may be particularly susceptible due to their specific immune defect (e.g., Aspergillus and other fungi in patients with chronic granulomatous disease [CGD]). Children should sleep in their own beds, preferably with rooms of their own, and keep away from individuals with obvious serious respiratory or other infections.

NUTRITION AND GASTROINTESTINAL FUNCTION

The height and weight of children with immunodeficiency should be documented at 3- to 6-month intervals on a standard growth chart. Adults should be weighed regularly as well. A falling off of growth or an absolute weight loss is an ominous feature of immunodeficiency and suggests that infections are not under good control or that other medical complications (e.g., hyperthyroidism, chronic diarrhea, malignancy) are present. Chronic antibiotic therapy may decrease appetite or cause diarrhea. Febrile episodes with severe chronic infections will decrease appetite, increase metabolic demands and result in slow growth.

Nutritional assessment, including a calorie count, may be indicated, and a dietary supplement might be tried. A multivitamin and mineral supplement is also advisable but only if levels of essential vitamins or minerals are shown to be low.

If there is noninfectious chronic diarrhea, a search for malabsorption, food intolerance, coeliac disease or hypovitaminosis is indicated. Symptoms of night blindness, proximal weakness, ataxia, or tremor should raise suspicions of secondary deficiencies of fat-soluble vitamins that are reversible on treatment (Ardeniz et al., 2008; Aslam et al., 2003; Aukrust et al., 2000).

Celiac disease may not necessarily present with frank malabsorption or even diarrhea; weight loss alone should lead to investigation of the gastrointestinal tract. However, partial villous atrophy is common and not just due to celiac disease. There should be a careful search for microbial agents (e.g., Giardia, Crytosporidia), enteropathic viruses (Annick et al., 2012), inflammatory bowel disease, the unexplained enteropathy of common variable immunodeficiency disorder (CVID) or structural abnormalities of the gastrointestinal tract such as intestinal lymphangiectasia.

Many immunodeficiencies are associated with autoimmune illnesses (Lehman and Ballow, 2008) or hepatic nodular regenerative hyperplasia (Ward et al., 2008; Malamut, et al, 2008).). Since in the past, use of blood products, including intravenous immunoglobulin (IVIG), resulted in hepatitis C (Centers for Disease Control, 1994), liver function tests should be done at yearly intervals on all patients receiving Ig therapy, and more regularly (every 3–6 months) if there is a risk of hepatitis C, since early treatment has been shown to be effective (Chapel et al., 2001).

RESPIRATORY AND PULMONARY PROBLEMS

Sinusitis, bronchitis, and recurrent pneumonia are common in patients with most forms of immunodeficiency and are often the chief cause of morbidity and mortality (Quinti, et al., 2011; Lucas et al., 2010) Sinusitis must be suspected if the patient has any of the following: purulent nasal discharge, frontal headaches, chronic nasal obstruction, postnasal drip, a decreased gag reflex, or unexplained coughing or wheezing. A microbial diagnosis (including fungal cultures) should be sought. Waters-view sinus films are of value, particularly in youngsters, but a CT scan of the sinuses is used for definitive diagnosis in adults. Treatment consists of prolonged courses of antibiotics, nasal decongestants, and nasal steroids. Nasal polyps should be surgically removed; surgical sinus drainage may be indicated.

Many patients with a significant immunodeficiency have experienced pneumonia at one time or another prior to diagnosis and developed bronchiectasis. All breakthrough infections, particularly those in the chest, must be treated promptly. Vigorous exercise, including team sports, should be encouraged to facilitate expectoration of mucus and to prevent bronchial sepsis.

A chronic cough due to bronchiectasis or wheezing is cause for special concern; attempts to detect structural damage or reversible airways disease are essential. Under such circumstances pulmonary function tests are conducted, including the response to bronchodilator therapy.

If bronchiectasis is found on CT scan, regular physiotherapy and exercise should be instigated. If the patient has antibody deficiency, a trial of a higher-dose IVIG (600–800 mg/kg) might be used (Lucas et al., 2010) and has been shown

to improve pulmonary function (Bernatowska et al., 1987; Eijkhout et al., 2001; Roifman et al., 1987). For older patients, routine pulmonary function testing at 6-month or yearly intervals is recommended for all but the mildest immunodeficiency syndromes.

Patients who develop pulmonary infiltrations not responsive to antibiotics should undergo bronchoalveolar lavage or lung biopsy for an exact microbiological or tissue diagnosis. Lung biopsy may reveal nonmicrobial granulomatous disease (Mechanic et al., 1997; Viallard et al., 1998; Wislez et al., 2000), lymphoid interstitial pneumonitis (LIP) (Davies et al., 2000; Wheat et al., 2005, Chapel and Cunningham-Rundles 2009), or even frank lymphoma (Dhalla et al 2011).

Immunodeficient individuals with chronic lung disease should be started on a program of pulmonary therapy including postural drainage, the use of a flutter device or oscillatory vest to loosen secretions, and inhaled bronchodilators or other agents (Atrovent, cromolyn, antibiotics). It is particularly important to identify patients likely to develop chronic lung disease early, so that periodic assessment of pulmonary function, management of sinus disease, and vigorous treatment of early pulmonary infections can be undertaken. Lung abscesses must be treated surgically. Some patients with neutrophil dysfunction, partial antibody deficiencies, or selective IgA deficiency may also have IgE-mediated allergies and may benefit from inhaled corticosteroids and oral antihistamines.

SKIN PROBLEMS

Patients with immunodeficiency often have cutaneous abnormalities (telangiectasia, molluscum contagiosum, warts, eczema, etc.). The most common cutaneous complication is chronic skin infection, particularly in the hyper-IgE immunodeficiency syndromes and in certain T-cell deficiencies, infections such as mucocutaneous candidiasis. These patients require local and systemic antibiotics and/or antifungal therapies, usually prophylactically.

Chronic eczema is a common problem in the immunocompetent population, but this may be more severe or may become infected in the immunodeficient patient. It is a cardinal feature of Wiskott-Aldrich syndrome (WAS). Local steroids, tacrolimus or picrolimus ointments, and antipruritic medications are the mainstays of therapy. If there is cracking, weeping, or regional lymphadenopathy, antibiotics, either local or systemic, should be used. Clarithromycin is particularly well tolerated in chronic eczema.

Severe warts are not uncommon (Barnett et al., 1983) and often refractory to usual methods of control. Subcutaneous IG therapy was reported to be of benefit in one child with common variable immunodeficiency disorder (CVID) and warts (Lin et al., 2009). The presence of extensive warts may indicate the presence of a rare syndrome, warts, hypogammaglobulinemia, infection, myelokathexis (WHIM)

Alopecia or vitiligo are also relatively common in antibody-deficient adults with CVID; both of these distressing conditions are untreatable.

Subcutaneous granulomas (as well as pulmonary and gastrointestinal granulomas) are not uncommon in CVID. There are several reports suggesting that these granulomas respond to anti–tumor necrosis factor (TNF) therapy with etanercept (Lin et al., 2006; Smith and Skelton, 2001) or infliximab (Hatab and Ballas, 2005, Thatayatikom et al., 2005), though with disappointing results on granuloma in other sites (Thatayatikom et al., 2005).

VACCINES

A review of immunization strategies in special clinical circumstances is provided in the "Red Book" (American Academy of Pediatrics, 2012); each country has a similar source of advice depending on the level of risk for a particular infectious disease.

Live attenuated vaccines, including, poliomyelitis, rotavirus, measles, mumps, rubella, bacillus Calmette-Guérin (BCG), and varicella/herpes zoster, should be avoided in all patients with severe antibody or cellular immunodeficiencies because of the risk of vaccine-induced infection. The evidence from DiGeorge syndrome patients and those with HIV suggest that it is safe to give live-virus vaccines to those with CD4 counts above 400 cells/uL (Perez et al., 2003). These vaccines have also been given without sequelae to patients with selective IgA deficiency, mucocutaneous candidiasis with intact cellular immunity to other antigens, and phagocytic and complement immunodeficiencies and to children fully reconstituted following human stem cell transplantation (HSCT).

Paralytic poliomyelitis, chronic encephalitis, and prolonged poliovirus shedding from the gastrointestinal tract were recognized complications of attenuated live poliomyelitis vaccination (Sabin) in immunodeficiency (Wyatt, 1973), although this vaccine is rarely used now since the World Health Organization implemented its strategy to eliminate poliovirus. In countries where oral polio vaccine is used, parents, siblings, and other household members of an immunodeficient child should be given inactivated poliomyelitis vaccine because of the risk of spread to the patient of live vaccine-strain oral virus.

Other routine inactivated vaccines should be given to patients with adequate antibody immunity, including yearly influenza vaccine. We also administer killed vaccines to patients with partial antibody deficiencies (e.g., IgG subclass deficiency, ataxia-telangiectasia). The antibody response to these vaccines can also be used to assess their B-cell function. Many such patients have a short-lived antibody response that subsequently decreases over 6 to 12 months.

Vaccines (except for oral attenuated poliovirus and rotavirus) should also be given to family members of patients with immunodeficiency. Adult individuals with a propensity to lower or upper respiratory infection but some antibody function should be given pneumococcal polysaccharide vaccine, and conjugated pneumococcal vaccine to such children. For patients with intact cellular immunity, periodic tuberculin testing or an ELIspot test for mycobacteria-induced interferon (IFN)-γ production may be indicated, particularly if corticosteroid or anti-TNF therapy is anticipated.

ANTIBIOTICS AND ANTIVIRALS

Antibiotics are life-saving in the treatment of infectious episodes of patients with immunodeficiency. The choice and dosage of antibiotics for specific infections are identical to those used in normal subjects, but treatment must be started early and last longer. Culture specimens may be obtained prior to therapy; these will be especially important if the infection does not respond promptly to the initial antibiotic chosen.

Patients should be preemptively treated for infectious episodes pending cultures, even if this necessitates frequent or chronic use of antibiotics and occasional unnecessary hospitalizations. If the infection does not respond promptly to antibiotics, the physician should consider the possibility of fungal, mycobacterial, viral, or protozoal (*Pneumocystis jiroveci*) infection. Invasive procedures such as bronchoalveolar lavage or biopsy may be necessary.

Antiviral therapy can be used effectively in many immunodeficiencies. Exposure to influenza or early symptomatic influenza infection may be managed with amantadine or ramatidine or the neuraminidase inhibitor drugs zanamivir and oseltamivir. Severe herpes simplex infection, chickenpox, or herpes zoster should be treated with aciclovir. Ribavirin aerosols have been used in the treatment of respiratory syncytial virus (RSV) and parainfluenza viral infections occurring in severe immunodeficiencies but are not effective in enterovirus infections (McIntosh et al., 1984). Aciclovir can also be used in the incubation period to modify or prevent chickenpox after exposure or as soon as the first lesion appears (Asano et al., 1993). Topical cidofovir has been used successfully to treat severe molluscum contagiosum (Davies et al., 1999).

Antibiotics should be given prophylactically with each dental or surgical procedure. Amoxicillin and gentamicin can be given intravenously 1 hour before and 8 and 18 hours after major surgery, or 3 days of oral broad-spectrum antibiotics can be used for less serious procedures (e.g., dental procedures).

ANTIMICROBIAL PROPHYLAXIS

Continuous prophylactic antibiotics often are of benefit in immunodeficiencies and are essential in those who have been splenectomized. They are especially useful in disorders characterized by rapid, overwhelming infections (e.g., complement deficiencies or in WAS patients who are not receiving immunoglobulin therapy). They can also be used in patients with antibody deficiencies, especially in those with structural damage, when recurrent infections occur despite optimal Ig therapy, and in phagocytic disorders in which no other form of therapy is available.

If respiratory tract infections are the highest risk, penicillin, ampicillin, or dicloxacillin given orally, 0.5 to 1.0 g/day in divided doses, may be recommended. The use of prophylactic antibiotics in CVID is controversial and is usually reserved for those with severe bronchiectasis in whom the risk of progression due to recurrent pneumonia is substantial. Azithromycin once daily (5 mg/kg, maximum of 500 mg/day or on alternate days or two or three times weekly) is often effective; its longer half-life and its anti-inflammatory action make this a popular choice.

Table 59.2 **RECOMMENDATIONS FOR PROPHYLAXIS AGAINST *PNEUMOCYSTIS JIROVECII* IN HIV AND PRIMARY IMMUNODEFICIENCY ON THE BASIS OF CD4 LYMPHOCYTE COUNT**

Age	CD4 COUNT*
<12 months	<1,500 cells/μL
12–23 months	<750 cells/μL
2–5 years	<500 cells/μL
>5 years	<200 cells/μL
Adults	<200 cells/μL

* Level at which prophylaxis is indicated:

- CD4 count <25% of total lymphocytes
- Past history of Pneumocystis *jirovecii* infecion
- Children with X-linked hyper-IgM syndrome (CD40L mutations)
- All patients with severe combined immunodeficiency

Prophylactic antibiotics alone, without IG therapy, are often used in patients with less severe antibody deficiencies (e.g., selective IgA deficiency, IgG subclass deficiencies, partial antibody deficiencies, transient hypogammaglobulinemia of infancy) and recurrent respiratory infections.

P. jiroveci pneumonia (PCP) prophylaxis is recommended for children with significant primary and secondary cellular (T-cell) immunodeficiencies (Centers for Disease Control, 1991) (Table 59.2). Prophylaxis consists of trimethoprim-sulfamethoxazole (TMP-SMX), 160 mg/M2 of body surface per day of the TMP and 750 mg/M2 per day of SMX given orally in divided doses twice per day three times per week, with frequent checks of the leukocyte count. Alternative drugs for PCP prophylaxis include pentamidine, dapsone, and atovaquone.

IMMUNOGLOBULIN (IG) THERAPY

IG therapy is indicated only for those patients with proven antibody deficiency (Table 59.3). Ig is not of value in the treatment of cellular, phagocytic, or complement immunodeficiencies. It is of only limited value in combined antibody and cellular immunodeficiencies, although it is used routinely prior to hematopoietic stem cell transplantation (HSCT) for Severe Combined Immune Deficiences [SCID] and also in those HSCT recipients in whom only the T-cell system is reconstituted.

Three human IG formulations are available: intravenous immunologlobulin (IVIG), concentrated liquid products for intramuscular use (IMIG), and subcutaneous immunoglobulin (SCIG). The route selected depends on ease of venous access, the dose and interval needed to keep the patient free of infection and the patient's preference and therefore compliance.

IG is derived from plasma pooled from 10,000 to 50,000 donors; the individual donors are first screened for pathogens, including hepatitis B and C viruses and HIV. The pool is treated with cold ethanol (Cohn fractionation) that precipitates the gammaglobulin fraction (termed fraction II); this

Table 59.3 PRIMARY IMMUNODEFICIENCIES IN WHICH HUMAN INTRAVENOUS OR SUBCUTANEOUS IMMUNOGLOBULIN MAY BE BENEFICIAL

Antibody Deficiencies:
X-linked and autosomal agammaglobulinemias
Common variable immunodeficiency disorders
Hyper-IgM syndromes
Transient hypogammaglobulinemia of infancy (in selected cases)
IgG subclass deficiency, with or without IgA deficiency (in selected cases)
Antibody deficiency with normal serum immunoglobulins (in selected cases)
Combined Deficiencies:
Severe combined immunodeficiencies (all prior to BMT and after BMT, if reconstitution of B cell function has failed)
Wiskott-Aldrich syndrome (if associated with severe immunodeficiency)
Ataxia-telangiectasia (if antibody-deficient)
Short-limbed dwarfism or cartilage-hair hypoplasia
Bare lymphocyte syndromes
X-linked lymphoproliferative syndromes (possible benefit)
22q11 deletion syndromes (if severe antibody deficiency)
Hyper-IgE syndromes (if severe antibody deficiency)
NEMO

BMT, bone marrow transplantation.

Table 59.4 PREPARATION OF INTRAVENOUS IMMUNOGLOBULIN FROM COHN FRACTION II*

1. Physical removal of aggregates by ultracentrifugation or gel filtration
2. Treatment with proteolytic enzymes
3. Treatment with chemicals that reduce sulfhydryl bonds, followed by alkylation of the free SH bonds
4. Addition of disaccharides or other stabilizing agents (e.g., glycine, proline)
5. Incubation at low pH

*These methods are used to eliminate high-molecular-weight complexes and most viruses.

precipitate undergoes several additional steps to concentrate the IG, remove other serum proteins and high-molecular-weight complexes, and remove or inactive pathogens. It is then treated by solvent-detergent (or other chemicals), by pasteurization or by nanofiltration as additional steps to ensure viral inactivation or virus removal (Henin et al., 1988; Stiehm et al., 2008) (Table 59.4).

The final product is again tested for pathogens, pyrogens, concentration of IgG, IgA and IgM as well as selected antibody levels. It is then brought to a relevant concentration for subcutaneous or intramuscular use (15–25 percent) or intravenous use (5–10 percent).

IG contains a wide spectrum of antibodies to viral and bacterial antigens (Stiehm et al., 2008). In most IG preparations is more than 95 percent IgG, but trace quantities of IgM and IgA are present; there is currently one preparation that contains higher concentrations of IgM and IgA but this is in limited supply. The IgM and IgA globulins are usually therapeutically insignificant because of their short half-lives (<7 days) and their low concentrations. IG contains all IgG subclasses and multiple IgG allotypes (Gm and Km types).

IVIG THERAPY

IVIG is the most widely used treatment for the majority of primary and secondary immunodeficiency syndromes in N. America though the proportion in relation to SCIG is more even in other continents, particularly in Europe. Over 20 IVIG preparations from different manufacturers have been used worldwide; all are slightly different in terms of their formulation (due to patent law) and therefore may have minimally different risks. In practice, all of these preparations have acceptable serum half-lives (18–28 days), contain all IgG subclasses, have minimal anticomplementary activity, have a good and diverse antibody content, and are negative for hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV), and HIV.

There are advantages and disadvantage to the administration of IG by the intravenous route compared to the subcutaneous or intramuscular route (Table 59.5). Dosage, methods of administration, and adverse reactions are discussed in the later section devoted to antibody deficiencies.

SCIG THERAPY

IG for subcutaneous use is also a sterile (15–25 percent, 150–200 mg/mL), solution without preservative and is now available in many countries. Its advantages and disadvantages are shown in Table 59.5. A slow subcutaneous infusion of 10 to 12 percent IVIG was given in the United States prior to the licensure of a 16 percent solution of SCIG in 2006 (Stiehm et al., 1998; Welch and Stiehm, 1983).

Dosage, methods of administration, and adverse reactions of SCIG are also discussed in the chapters on antibody deficiencies.

IGIM THERAPY

Whereas most IG preparations for intramuscular injection in the United States had thimerosal as a preservative and were therefore unsuitable for subcutaneous or higher-dose intramuscular use, products suitable for subcutaneous infusion have been available in Europe and elsewhere for decades, obviating the need for intramuscular preparations.

Intramuscular products contain high-molecular-weight complexes that are strongly anticomplementary; these may account for the occasional systemic reaction to IGIM that is inadvertently injected intravenously. These products should not be given intravenously.

Table 59.5 ADVANTAGES AND DISADVANTAGES OF INTRAVENOUS IMMUNOGLOBULIN OVER SUBCUTANEOUS IMMUNOGLOBULIN

Advantages:
Ease of administering large doses
Less frequent treatments
More rapid action
No loss in tissues from proteolysis
Avoidance of swelling at injection sites
Disadvantages:
Requires venous access
Requires more training for home or self-infusions
Longer duration of infusion
Higher risk of serious side effects

IGIM therapy was utilized regularly for antibody deficiencies until the early 1980s, when IVIG therapy was introduced. The initial recommended dose was 100 mg/kg per month, based on studies of the Medical Research Council of the UK (1969). Treatment was given weekly at a dose of 25 mg/kg because of the large volumes needed (50 mL/month for a 70-kg adult).

These injections are painful and have occasionally resulted in sterile abscesses at the site of injections (Haeney et al., 1979). One case of mercury toxicity was reported as a result of the mercury used as a preservative (Matheson et al., 1980). Adverse events were relatively uncommon, other than the local reactions, though occasional anaphylactoid reactions and even fatalities were reported. The amount of IG that can be administered intramuscularly is limited, and serious breakthrough infections may occur as a result. This route is rarely used today, since IVIG and SCIG are more widely available and are now included in the WHO Essential Medicines lists for adults and for children.

HEPATITIS C IN IVIG

Hepatitis C virus (HCV) was transmitted through a number of IVIG lots prior to 1995 (Bjorkander et al., 1988; Bjoro et al., 1994; Centers for Disease Control, 1994; Christie et al., 1997; Lever et al., 1984; Ochs et al., 1985; Razvi et al., 2001; Schiff, 1994; Williams et al., 1989; Yap et al., 1994; Yu et al., 1995). Once testing for HCV became available, such transmission has not been reported including no reports relating to subcutaneous use. IG now is only manufactured with units tested for HCV by polymerase chain reaction (PCR), and all batches undergo viral inactivation steps (Stiehm et al., 2008).

These HCV cases followed the exclusion of plasma containing antibodies to HCV prior to the PCR assay. Because HCV infection does not result in detectable antibodies for up to 6 months after exposure in immunocompetent individuals (the window period), unchallenged virus from a few viremic donors contaminated a few of the final batches (Yu et al., 1995). Over 200 patients worldwide became infected (Centers for Disease Control, 1994; Christie et al., 1997; Healey et al., 1996; Schiff, 1994; Yu et al., 1995). The Gammagard/Polygam preparations involved in the outbreak were replaced with Gammagard-SD and Polygam-SD, whose manufacture included a solvent-detergent treatment to inactivate HCV and other membrane-enveloped viruses.

In a previous outbreak, Bjoro et al., (1994) reported that immunocompromised patients had a severe and rapidly progressive course of HCV infection, and the responses to IFN were poor. However, this was a study of patients with chronic infection of undefined length and unknown or mixed genotypes of HCV. The 1994 HCV outbreak in the United Kingdom provided a unique opportunity to study the long-term rate of progression of HCV and responses to therapy in patients with known onset of infection and a single genotype, genotype 1a (Christie et al., 1997; Healey et al., 1996). With early (acute-phase) high-dose IFN-α therapy, over half the patients cleared the virus and remained clear at 5 years (Chapel et al., 2001). Furthermore Razvi et al., (2001) reported that 16 of 58 (27 percent) immunodeficient patients who developed HCV infection from IVIG resolved their infection; this rate of recovery was the same as that in IFN-α–treated patients and those receiving no therapy.

There have been no further reports of HCV infections transmitted by IVIG since 1994. Manufacturers of IVIG are now required to show that the virus inactivation processes eliminate HCV. These studies have substantiated the effectiveness of low pH, pepsin, solvent-detergent, caprylate precipitation, pasteurization, and nanofiltration to remove HCV. Nevertheless, they do not guarantee the complete absence of other known or unknown infectious viruses.

No cases of HIV transmission have been identified with the use of IVIG, and HIV is readily inactivated by cold ethanol fractionation or partitioned into fractions other than Cohn fraction II.

PRION DISEASE AND IVIG

Several IVIG lots derived from plasma obtained from donors subsequently found to be at risk for Creutzfeld-Jakob disease (CJD) were recalled, although no cases of CJD or variant CJD (vCDJ) associated with mad cow disease transmitted by IVIG have been reported. (Zou et al., 2008).

Vigilance is needed in this area as for other potential blood borne pathogens, and the International Union of Immunological Societies (IUIS) Committee for Primary Immunodeficiency Diseases encouraged early referral to the Edinburgh Surveillance Unit of immunoglobulin recipients, especially those on long-term, repeated therapy, if they develop unexplained neurological or psychiatric symptoms. Ziegner et al., (2002) have reported unexplained neurodegeneration in 14 immunodeficient patients treated with IVIG in the United States and Europe, although none of these patients had proven prion disease and several may have been vitamin A deficient (Aslam et al., 2004).

Currently, British plasma and that from individuals who have lived in Great Britain for more than 3 months between

1980 and 1996 or who have stayed in Europe for an accumulative time of more than 5 years since 1980 are excluded from donating blood for use in immunoglobulin production. Several countries, including the United States use only plasma collected nationally, in line with the WHO guidelines for self-sufficiency but this is increasingly difficult as larger amounts of plasma is needed to meet the increasing demand for IG therapies, including for immunomodulation.

HIGH-TITER IGs

Following chickenpox or herpes zoster exposure of the immunodeficient child, varicella-zoster immune globulin (VZIG) may be given to prevent chickenpox. Alternatively, or in combination, oral aciclovir can be used. For antibody-deficient children receiving regular IVIG infusions at monthly intervals, VZIG is not necessary, since IVIG contains antibody to varicella/zoster virus. Even if not prevented, varicella infections are markedly attenuated by IVIG (Stiehm and Keller, 2009).

RSV-IG (RespiGam), licensed in 1996 in the United States, was a high-titer human IVIG that prevented or modified RSV infections in high-risk infants, such as those born prematurely and infants with bronchopulmonary dysplasia (Groothuis et al., 1993), during the respiratory virus season (November–April). RespiGam was replaced in 2003 with monthly intramuscular injections of the RSV monoclonal antibody palivizumab (Synagis, 15 mg/kg) (American Academy of Pediatrics, 2012). Immunodeficient infants less than 2 years of age should also receive palivizumab, even if they are on IG replacement therapy, since IVIG or SCIG has negligible titers of RSV antibody.

The prophylactic use of other high-titer immunoglobulins such as tetanus immunoglobulin (TIG) or hepatitis B immunoglobulin (HBIG) following exposure to those agents is particularly important in the immunodeficient patient who is unable to make antibodies (Stiehm and Keller, 2009).

CORTICOSTEROIDS

Corticosteroids are occasionally necessary in patients with immunodeficiency for such problems as asthma, LIP, obstructive gastrointestinal or genitourinary tract granulomas, hemolytic anemia, immune thrombocytopenia, severe contact hypersensitivity reactions, or graft-versus-host disease. Short-term courses are well tolerated; if chronic or high-dose corticosteroid therapy is used, *P. jiroveci* prophylaxis should be considered, particularly in those patients with low CD4 counts or T-cell deficiency.

We have used inhaled corticosteroids in children with CGD and local steroids for eczema in WAS and other immunodeficiencies without complications. Prolonged high-dose corticosteroids will severely depress the lymphocyte count, including that of T- and B-cell subpopulations, and may cause hypogammaglobulinemia, even in normal children, although lymphoproliferative and antibody responses usually remain intact (Lack et al., 1996).

PRENATAL MANAGEMENT

Pre-implantation diagnosis is available in some countries for those women known to be carriers of a specific severe immunodeficiency. Prenatal or diagnosis by newborn screening is of value if early treatment, therapeutic abortion, or Cesarean section is a possibility. In utero tissue typing of the fetus is sometimes of value to identify a potential matched related or unrelated HSCT donor, or an umbilical cord transplant donor (Vowels et al., 1993). In utero haploidentical BMT has been accomplished but is not routinely available (Bartolome et al., 2002; Flake et al., 1996; Merianos et al., 2008).

If a previous sibling has had a severe T-cell immunodeficiency and the mother is pregnant again, a number of options must be considered and planning is imperative.

Prenatal diagnosis should be considered either by genetic testing or phenotyping of a fetal blood sample for lymphopenia or thrombocytopenia. Fetal sampling can be done as early as 18 to 20 weeks. If the molecular defect is known through gene analysis of affected family members, prenatal diagnosis can be performed by analyzing fetal DNA obtained by chorio-villous biopsy (CVS) or by collecting amniocytes.

Cesarean section may be performed if a difficult labor is anticipated, but it is not routinely indicated. Cesarean section prior to the onset of labor is recommended when neonatal thrombocytopenia is anticipated, such as in WAS, to prevent a cerebral bleed.

NEONATAL MANAGEMENT

The importance of early diagnosis and treatment of primary immune deficiency to prevent death or long-term complications cannot be overemphasized (Griffith et al., 2009). Thus newborn screening, as employed in many countries for SCID, will soon be extended throughout the developed countries.

Cord blood should be obtained at delivery from newborns suspected of having an immunodeficiency. For optimal collection, a trained person should be available to drain the placenta for maximal yield, collect the blood in a sterile fashion, and anticoagulate it in multiple aliquots. Cord blood should be tested immediately for blood count, immunoglobulin assays, B- and T-cell enumeration, phytohemagglutinin and/or allogeneic cell stimulation, and human leukocyte antigen (HLA) typing. The remaining cord blood can be cryopreserved for possible future gene therapy or, if the infant is unaffected, for possible use in a future affected sibling.

Newborns at risk for severe combined immunodeficiency (SCID) should remain in a protective environment with reverse isolation or in an isolator until their immunological status is clarified or definitive treatment completed. On no account should such an infant be immunized with live vaccines; BCGosis is an inevitable complication of BCG immunization and is not only difficult to treat but increases the risk of failure of a subsequent HSCT.

A chest X-ray for thymic size should be done as soon as possible; an absent thymic shadow in an unstressed newborn is highly suggestive of a T-cell immunodeficiency, although ill infants from any cause may have thymic involution. Gastrointestinal sterilization with nonabsorbable antibiotics may be considered.

Pneumocystis prophylaxis (with TMP-SMX) should be considered for all patients with severe T-cell deficiencies or a CD4 cell count below 1,500 cells/uL, which is less than the fifth percentile for their age group (Stiehm et al., 2004).

FAMILY STUDIES

Immunoglobulin and complement levels should be obtained in the immediate family of all patients with antibody or complement deficiencies to determine a familial pattern. If other family members have suggestive histories, they should also be studied. Newborn siblings of an affected patient are followed carefully from birth for manifestations of a similar disorder. In disorders in which procedures are available for heterozygosity testing, the parents, siblings, and children of the affected subject can be tested for future genetic counseling.

SUPPORT GROUPS

Participation in support groups (listed at end of this chapter) provides essential information and help for patients and their families with schooling, insurance, access to medical care, and so forth. Medical and nursing teams can provide only limited resources to help families cope with the social, economic, emotional, and psychological issues that these diseases impose on families. Other patients and families can offer a perspective and mutual support not obtainable from medical personnel.

PART II: SPECIFIC IMMUNODEFICIENCIES

ANTIBODY DEFICIENCIES

Since many antibody-deficient patients have a reasonable life expectancy with IG therapy (Chapel et al., 2008), other general measures as outlined in the first section are particularly important in ensuring a long and active life. This includes lifestyle counseling (no smoking, good diet, exercise), prompt treatment of infection, avoidance of contagious situations and sick contacts, and recognition and treatment of disease-related complications such as granulomas, autoimmunity and malignancy.

IG Replacement Therapy

In patients with severe symptomatic antibody deficiency (Table 59.3), such as X-linked agammaglobulinemia (XLA), CVID or one of the hyper-IgM syndromes, human IG replacement is the mainstay of therapy.

Like the well-controlled diabetic on insulin therapy, many patients on adequate regular IG replacement are able to live relatively symptom-free. However, some antibody deficient subjects given IG remain chronically ill or undergo a progressive downhill course. Those with XLA and pre-existing bronchiectasis have long-standing pulmonary difficulties, as do those CVID patients who suffered pneumonia before diagnosis (Quinti et al., 2007, Chapel et al., 2008). CVID patients may also have immunological or hematological deficits as well as antibody deficiency or chronic lung or gastrointestinal disease (Chapel et al., 2008, 2009, 2012)

Patients with IgA or IgG subclass deficiency, transient hypogammaglobulinemia of infancy, or selective Ig antibody deficiency need not be treated with IG unless there is chronic refractory infection and/or a documented inability to produce specific antibodies following immunization (Moschese et al., 2008; Stiehm, 2008).

The aim of IG therapy is to keep the patient free of bacterial infections by maintaining serum antibodies at protective levels. Serum trough IgG concentration is used as a surrogate and the IgG level kept within the normal range in addition to monitorin of any breakthrough infections. However, as found in healthy populations, each individual needs a different level of trough IgG to maintain prevention against bacterial infections (Lucas et al., 2010).

IG is a blood product and therefore used only in patients in whom the risk/benefit analysis is likely to be beneficial to the patient. Pooled plasma from 10,000 to 50,000 donors is fractionated, and the fraction containing IG is then purified and treated with antiviral steps to provide a final product at various concentrations, with a variety of stabilizing agents, depending on the manufacturer (Table 59.4).

IVIG Dosage

Several studies indicate that larger doses of IVIG (400–800 mg/kg) are superior to lower doses (100–300 mg/kg per month) in terms of subjective improvement, reduced hospitalization rates, improved chest X-rays or CT scans, normalization of pulmonary function, and decreased incidence of major or minor infections (Bernatowska et al., 1987; Garbett et al., 1989; Haeney, 1994; Liese et al., 1992; Montanaro & Pirofsky, 1984; Ochs et al., 1984; Roifman et al., 1987; Stiehm, 1997; Wood et al., 2007; Orange et al., 2010, Lucas et al., 2010).

A common practice is to start IVIG at 400 mg/kg at 2- to 4-week intervals but to give additional doses (loading) at the onset of therapy to raise the IgG level quickly. After initiation of IVIG, the IgG trough level increases over several months as the tissue spaces become saturated with IgG. After 3 months the clinical progress of the patient alongside the preinfusion IgG level is assessed and the dose adjusted (Lucas et al 2010; Orange et al., 2006; Stiehm, 1997).

Those antibody deficient patients with chronic lung disease, vasculitis, rheumatic complaints, chronic diarrhea, protein-losing enteropathy or failure to thrive may require a higher dose (e.g., 800–1,000 mg/kg per month) to control disease manifestations (Lucas et al., 2010; Eijkhout et al., 2001; Roifman et al., 1987).

Administration of IVIG

IVIG administration requires venous access, which is sometimes a problem in small children, obese or elderly patients. The local use of an analgesic cream or spray applied to the infusion site prior to venipuncture makes the infusions more acceptable.

Close monitoring is required, especially during the first few infusions, when the risk of adverse reactions is much higher.

These can be minimized or prevented by premedication (see below). Adverse reactions are infrequent when regular IVIG infusions are given and once the patient is stable, provided the correct rates for infusions are observed or the patient has not had a breakthrough infection, adverse reactions are uncommon and nearly always mild; no serious reactions were reported in the large UK audit. (Brennan et al., 1995).

In Europe and the United States, IVIG infusions have been shown to be sufficiently safe to enable selected patients (adults and children) to receive infusions at home with the help of either a home infusion service, a responsible adult family member or by self-infusion (Kobayashi et al., 1990). Self-infusion without another informed adult in the house is discouraged. In one study of patients selected to self-infuse at home, the number of adverse reactions was 0.1 percent; universally the few reactions that did occur were mild (Brennan et al., 1995). Parents, patients and nurses who perform home infusions must be taught to recognize and treat adverse reactions, and their competency must be documented before such schedules are put in place.

In patients who have received infusions without adverse events, self- or parent administration at home can be accomplished with great cost savings in some countries, depending on the health-care system (Ashida and Saxon, 1986; Kobayashi et al., 1990; Ochs et al., 1986; Sorensen et al., 1987). This type of therapy is used by many patients in the United Kingdom and in other parts of Europe. In the United States, most IVIG infusions are performed in a clinic or by home infusion personnel.

An IVIG infusion usually requires 2 to 4 hours. The initial rate is 0.5 mg/kg/min (0.01 mL/kg/min of the 5 percent products), and this can be doubled at 20- to 30-minute intervals if there are no side effects to a maximal rate of 2 to 3 mg/kg/minute (0.04–0.06 mL/kg/minute). Ten percent products have been introduced and even faster rates have been shown to be safe, although each product has an individual recommendation. Adverse events tend to be associated with rapid infusion rates in patients with concurrent acute infections or previously untreated patients, or when a significant time between infusions has transpired (>6 weeks) (Brennan et al., 1995).

Side Effects of IVIG

Typical reactions associated with IVIG infusions include headaches, nausea and vomiting, flushing, chills, myalgia, arthralgia, and abdominal pain (Duhem et al., 1994; Katz et al., 2007; Ochs et al., 1980; Orange et al., 2006; Stiehm, 1997). Less commonly chest tightness, hives or even anaphylactoid reactions can occur. Severe, life-threatening reactions are very rare.

Immediate minor reactions can be avoided or diminished by slowing the infusion rate. Patients with minor side effects such as headaches, shaking chills, nausea and vomiting, or myalgia/arthralgia can be treated (and for subsequent infusions pretreated) with oral acetaminophen or a nonsteroidal anti-inflammatory drug and antihistamines. Severe reactions are treated with antihistamines and corticosteroids. The need for parenteral epinephrine is very rare and is no longer recommended for self-infusion patients who are well established on IG therapy, due to the risk of misuse.

If a severe reaction is anticipated (on a first infusion in a patient with marked bronchiectasis, for example), hydrocortisone (6 mg/kg, maximum 150 mg) can be given intravenously 1 hour before infusion in addition to oral acetaminophen and antihistamines. These drugs can be repeated after 3 or 4 hours if the infusion is not finished. Occasionally switching to a different IVIG product may alleviate recurrent reactions.

Serious late but very rare adverse reactions are almost always confined to high-dose usage of IVIG in immunomodulating doses (Duhem et al., 1994). These include aseptic meningitis (Kato et al., 1988; Vera-Ramirez et al., 1992), thrombosis or disseminated intravascular coagulation (Comenzo et al., 1992; Woodruff et al., 1986), renal (Miller et al., 1992) or pulmonary (Rault et al., 1991) insufficiency or hemolytic anemia (Brox et al., 1987). Sucrose-containing IVIGs are particularly likely to result in renal complications (Katz et al., 2007), so this is largely avoided now. Some IG product were reported to contain higher concentrations clotting factor XI, in one case in association with an increased risk of thrombosis even at replacement levels; this has not been a problem since the manufacturing processes have been changed (Funk et al 2013).

IVIG is contraindicated in patients who have had an anaphylactic reaction to IVIG or other blood products. IVIG is initially given with caution in patients who have high-titer or increasing amounts of anti-IgA antibodies (Burks et al., 1986; Rachid et al., 2011), as these patients may be at higher risk of a serious reaction (Rachid and Bonilla 2012).

Patients with an IgA deficiency in association with a profound IgG deficiency are not at increased risk for the development of anti-IgA antibodies or reactions to IVIG.

In the past there was some evidence that certain brands of IVIG may be more reactogenic. In one study of Kawasaki disease, the two IVIG brands used were at equivalence therapeutically, but one had a 12-fold (2 percent vs. 25 percent) increase in side effects (Rosenfeld et al., 1995). Such a discrepancy, however, has not held up in clinical practice.

A few investigators gave high concentrations of IVIG infused rapidly over 20 to 40 minutes; this rapid rate was tolerated by some but not all patients (Schiff et al., 1991) and is not recommended.

SCIG

Products for SCIG have been developed as an alternative to IVIG (Table 59.5). The monthly dose is the same as that for IVIG, starting typically at 400 mg/kg per month, in divided doses (Thepot et al., 2010). This can be given every 10 days, once or twice a week, or even daily, once the patient has been loaded; loading can be done intravenously or subcutaneously (Borte M, Quinti, I., Soresina, A., Fernández-Cruz, E., Ritchie, B., Schmidt, DS., McCusker, C. Efficacy and safety of subcutaneous vivaglobin® replacement therapy in previously untreated patients with primary immunodeficiency: a prospective, multicenter study. J Clin Immunol. 2011; 31:952–961.)

Initially, SCIG was given as a 16 percent formulation by slow subcutaneous infusion at a rate of 0.05 to 0.2 mL/kg/hour or 3 to 12 mL/hour, using a syringe driver as a pump (Abrahamsen et al., 1996; Berger et al., 1980) but this was not only inconvenient

but resulted in sterile abscesses; rapid infusion (up to 40 mL/hr) is now used worldwide (Gardulf et al., 1995, 2001; Hansen et al., 2002).

The SCIG injections are usually given into the abdominal wall and/or lateral thighs with the use of a battery-operated pump, though a gentle continuous push is fine too (Shapiro R. Subcutaneous immunoglobulin: rapid push vs. infusion pump in pediatrics. Pediatric Allergy and Immunology 2013; 24:49–53.) Multiple sites can be used simultaneously, particularly in older children and adults. More recently it was shown that using a new infusion area (e.g. changing from abdomen to thighs) increases the risk of local tissue reactions (Gardulf A,. Nicolay, U. Replacement IgG therapy and self therapy at home improve the health-related quality of life in patients with primary antibody deficiencies. Curr Opin Allergy Clin Immunol. 2006; 6:434–442.) These injections are well tolerated and enable the patient to receive the same or increased amounts of IG as given intravenously (Berger, 2004; Gardulf et al., 1991, 1995). They are especially valuable for self-administration at home in children and adults who cannot manage IVIG due to difficulty with venepuncture (poor venous access, fragile veins, etc.) (Gardulf et al., 1991, 2004, 2006; Nicolay et al., 2006; Ochs et al., 2006).

A multicenter study of the efficacy and safety of IVIG versus SCIG therapy showed no significant differences in rates of breakthrough infections or adverse reactions (Chapel et al., 2000). The IgG trough levels achieved subcutaneously were equivalent to that of a similar intravenous dose. These injections are particularly suitable for children and are well accepted by the parents (Berger et al., 2010; Abrahamsen et al., 1996).

A 20% preparation has been approved by the FDA in 2010 and by the European Commission in 2011 for subcutaneous use.

In countries or situations where no preservative-free 16 or 20 percent SCIG is available, 10 or 12 percent IVIG can be given subcutaneously (Stiehm et al., 1998).

SCIG is particularly suitable for individuals with poor venous access, those who have had severe immediate reactions or aseptic meningitis to IVIG, and those who have protein-losing illnesses in which IVIG is lost rapidly in the gastrointestinal tract or the kidneys (Rezaei et al., 2011).

IVIG for Enterovirus Encephalitis and Polymyositis

Chronic meningoencephalitis, polymyositis, or fasciitis due to disseminated enterovirus infection (usually ECHO virus) occurs in patients with XLA and, less commonly, in patients with CVID or CD40 ligand deficiency (Crennan et al., 1986; Cunningham et al., 1999; Erlendsson et al., 1985; Johnson et al., 1985; Lederman and Winklestein, 1985; McKinney et al., 1987; Mease et al., 1981; Prentice et al., 1985; Quartier et al., 2000; Schmugge et al., 1999; Wilfert et al., 1977).

PCR is required for establishing a viral diagnosis on the cerebrospinal fluid (Chesky et al., 2000). Since the introduction of higher doses of IVIG, the incidence of these infections has dropped dramatically, but they still may occur, even if patients receive adequate doses of IVIG (Misbah et al., 1992; Rotbart, 1990) or prior to a diagnosis of immunodeficiency.

Treatment of these patients with high-dose IVIG modifies the severity of infection and improves survival (Erlendsson et al., 1985; Mease et al., 1981; Prentice et al., 1985; Quartier et al., 2000), but failures have been reported (Crennan et al., 1986; Johnson et al., 1985). The response to IVIG may depend on the titer of neutralizing antibodies against the specific enterovirus in the preparation (Mease et al., 1981).

A review of published reports indicates that more affected patients treated with IVIG survived than those given IMIG or immune plasma; of those who did not receive IVIG, none survived (Stiehm and Keller, 2004). Intraventricular infusion of IVIG contributed to the eradication of the virus in some patients but not in others. Patients originally responding appropriately to high-dose IVIG may relapse after the high doses are discontinued (Mease et al., 1985)

An open trial of the antiviral drug pleconaril was only partially successful (Rotbart and Webster, 2001). With the advent of enteroviral PCR (Chesky et al., 2000) a definitive diagnosis is now possible, which should make future trials of new antivirals more robust.

IVIG for Other Viral Infections

IVIG has been advocated for the treatment of chronic Epstein-Barr virus (EBV) infection. In uncontrolled studies, selected patients have shown clinical improvement (Finberg, 1988; Tobi and Straus, 1985). It has been suggested that IVIG be used prophylactically in EBV seronegative infants and children with an X-linked lymphoproliferative syndrome (XLP) to prevent EBV infection. One EBV-sero-negative college student with XLP died from overwhelming EBV infection despite monthly IVIG infusions (Okano et al., 1991). It is particularly important to ascertain the underlying molecular diagnosis and to differentiate XLP from CVID (Morra et al., 2001).

High-dose IVIG (500 mg/kg/daily) may be of value in the treatment of immunodeficient patients with other viral diseases, including cytomegalovirus (CMV) infection, parvovirus-induced pure red cell aplasia (Kurtzman et al., 1989; Seyama et al., 1998), and respiratory virus infections such as parainfluenza or adenovirus (Stiehm et al., 1986).

Therapeutic immunoglobulin (150–300 mg/kg body weight) has been used orally in a few patients with antibody deficiency to stop enteroviral shedding following inadvertent live poliomyelitis immunization (Losonsky et al., 1985) or to treat rotavirus infections (Barnes et al., 1982).

Monoclonal Antibodies and Etanercept

Anti-TNF cytokine therapies have been used in a few CVID patients with life-threatening or serious disabling granulomas (Boursiquot et al. 2013, Bates et al., 2004; Chua, 2007; Hatab, 2005; Lin et al., 2006; Smith and Skelton, 2001; Thatayatikom et al., 2005). However corticosteroids remain the initial treatment of choice (Boursiquot et al, 2013) until the various immunopathogeneses of granuloma and lymphoid interstitial pneumonitis are unraveled.

There is limited experience with Rituximab and azathioprine in those CVID patients with lymphoid interstitial pneumonitis

and B cell follicles in the lung biopsy (Chase et al., 2013). Since the experience is limited and the outcomes are variable, an international register would be helpful (Chapel, 2009).

The successful use of rituximab (anti-CD20 monoclonal antibody) in CVID for immune cytopenias has been reported (Gobert et al., 2011, Cunningham-Rundles, 2008), but the long-term safety is not yet clear; there is an increased risk of infection in patients receiving more than one course, particularly if not on IG replacement therapy (Gobert et al 2011). The apparent efficacy and safety in immunocompetent patients are only partially reassuring.

Other Therapies

IG therapy alone is insufficient to maintain good health in antibody-deficient patients and other measures are needed, including prompt and longer courses of antibiotic therapy for any breakthrough infections and education of patients to seek help and therapies without delay for noninfective complications (Wood et al., 2007).

Continuous antibiotic therapy may be necessary to keep some antibody-deficient patients free of infection. In particular, adult patients with severe bronchiectasis or adults and children with bacterial overgrowth in the gastrointestinal tract may benefit from antibiotics. Some providers use full therapeutic doses of a cephalosporin, penicillin, clarithromycin, or TMP-SMX and usually change antibiotics at 2- or 3-month intervals. Others prefer alternate-day azithromycin on a continuous basis.

Some patients with antibody deficiencies have concomitant and usually transient neutropenia (e.g., XLA, immunodeficiency with hyper-IgM). High-dose IVIG (2 g/kg) may reverse persistent and clinically significant neutropenia and should be tried first. If this is ineffective, granulocyte colony-stimulating factor (G-CSF) can be given.

Attempts to stimulate antibody synthesis by immunological enhancing agents have been made with minimal success. Of particular interest was the use of PEG-IL-2, a preparation of IL-2 conjugated to polyethylene glycol (PEG). PEG-IL-2 was given to 15 CVID patients (Cunningham-Rundles et al., 1994, 2001). After 6 to 12 months of therapy, in vitro proliferative responses improved and antibody responses to a neoantigen developed (in 4 of 8 patients tested), but clinical benefit was minimal.

Isolated successes have been reported with cimetidine (White and Ballow, 1985) and retinoic acid (Saxon et al., 1993). Blys (BAFF), an agent that activates B cells, looked promising in animal experiments, but robust clinical trials have not materialised yet.

The management of complications is an important consideration, particularly for patients with CVID (Chapel and Cunningham-Rundles, 2009). Lymphoproliferative and serious autoimmune complications are usually treated with corticosteroids (with or without steroid-sparing drugs, including azathioprine, 6-mercaptopurine, ciclosporin, mycophenolate mofetil, or methotrexate) (Boursiquot et al 2013; Bates, 2004; Davies et al., 2000; Meyer et al., 2005; Misbah et al., 1992; Park et al., 2005). There are reports of anti-TNF therapies and hydroxychloroquine for granuloma or LIP in patients with severe clinical phenotypes of CVID (Thatayatikom et al., 2005; Yong et al., 2008), though successful mainly in skin granuloma.

Gluten-insensitive enteropathy is usually treated with the nonabsorbable corticosteroid budesonide or low-dose 6-mercaptopurine. New therapies are desperately needed, once the immunopathology is understood; it is encouraging that one such patient with documented enteropathic virus has responded well to Interferon-α (D.S.Kumararatne – personal communication) and another patient to bismuth therapy. It is most important to correct avitaminosis with replacement vitamins, especially the fat-soluble vitamins A, D, and E by injection if they are not absorbed orally (Ardiniz et al., 2008; Aslam et al., 2004; Aukrust et al., 2000).

Splenectomy for antibody-deficient patients with idiopathic thrombocytopenic purpura (ITP) or autoimmune hemolytic anemia poses a theoretical risk of overwhelming infection in patients who are noncompliant with prophylactic penicillin therapy. However a recent survey in Europe has shown that splenectomy does not worsen mortality in CVID and adequate immunoglobulin replacement therapy appears to play a protective role in overwhelming post-splenectomy infections (Wong et al., 2013).

Treatment of malignancies is the same as in immunocompetent patients except for patients with defective DNA repair (see Chapters 46,47,48).

In antibody deficiencies associated with drug treatment (e.g. IgA deficiency following phenytoin administration or hypogammaglobulinemia following gold therapy), discontinuing the drug may result in reversal of the disease, although in some cases the antibody deficiency persists indefinitely (Cunningham-Rundles, 2004; Snowden et al., 1996) and the patient may need IG therapy.

Prognosis

The prognosis has improved significantly for antibody-deficient patients (Chapel et al 2008; Chapel and Cunningham-Rundles, 2009). Early diagnosis, optimal IG therapy, prompt attention to infection, and management of complications are crucial. Factors leading to a more guarded outlook in general include coexisting T-cell deficiency (as in WAS, CVID with low CD4 cell numbers known as late-onset combined immune deficiency-LOCID (Malphettes et al., 2009), CD40 ligand deficiency, etc.), structural tissue damage (notably bronchiectasis), unexplained enteropathy, polyclonal lymphoid hyperplasia, malignancy, or poor compliance with treatment regimens.

CELLULAR IMMUNODEFICIENCIES

The cellular immunodeficiencies vary markedly as to their immunological severity and associated manifestations. Many are syndromic immunodeficiencies such as WAS and ataxia-telangiectasia. These patients need continuous attention to prevent infection and treat coexisting problems.

The increasing use of HSCT to treat SCID and other T-cell deficiencies has provided a cure for many of these patients (see Chapter 60) (Filipovich, 2008; Gennery and Cant, 2008).

Gene therapy has cured patients with X-linked SCID, adenosine deaminase (ADA) deficiency, and WAS (Chapter 61), but it remains experimental and is available in only a few research centers (Thrasher, 2008).

Precautions

Patients with a significant T-cell deficiency need preventive measures to avoid infective complications before attempting curative immunotherapy.

Vaccines: Immunization with live attenuated vaccines against mycobacteria or viruses is contraindicated in patients with a T-cell deficiency. In particular BCG and oral rotavirus vaccine must be avoided. Progressive enterovirus encephalitis, measles encephalitis, and mumps encephalitis have been recorded in undiagnosed T-cell–immunodeficient patients following routine childhood immunization. Additionally, siblings and other close contacts, such as house-mates or tutorial groups, should be immunized with inactivated polio vaccine (Salk vaccine) since live virus vaccine may be transmitted to the patient.

Blood products: Special care must be taken in administering blood products. Blood or blood-cell components given to patients with primary (and secondary) T-cell immunodeficiencies should be CMV-negative and must be irradiated with 27 Gy to prevent graft-versus-host reactions. Radiation of blood products is a simple procedure and can be accomplished rapidly in most blood banks. It is often done routinely for all premature infants and, in some centers, for all newborns.

Surgery: Tonsillectomy and adenoidectomy are rarely indicated. Splenectomy is contraindicated, except in unusual circumstances such as severe unresponsive immune thrombocytopenia or autoimmune hemolytic anemia, though recent data suggests that with modern management and IG therapy this can be a safe procedure (Wong et al., 2013). It has been used to treat the thrombocytopenia in WAS (Litzman et al., 1996) or X-linked thrombocytopenia. The addition of a phagocytic defect (absence of a spleen) to the already existing immunodeficiency increases the risk of sudden, overwhelming sepsis without affecting levels of memory B cells (Sanchez-Ramon et al., 2008).

Antimicrobials: Splenectomized and T-cell–deficient patients may benefit from the administration of broad-spectrum prophylactic antibiotics, as well as prompt treatment of every febrile illness with antimicrobials.

Patients with a significant T-cell deficiency are at risk for *P. jirovecii* infections. Those over age 5 years with CD4 counts below 200/uL are candidates for PCP prophylaxis, and for patients less than age 5, PCP prophylaxis should be considered at higher CD4 count levels (Stiehm et al., 2004) (Table 59.3).

Prophylactic antifungal medications, such as oral nystatin, ketoconazole, fluconazole, itraconazole, and caspofungin (Cancidas), are used to prevent or treat chronic candidiasis and other fungal infections.

Continuous antiviral therapy is sometimes needed for patients with symptomatic chronic CMV or other herpes infections, including herpes simplex or varicella. RSV infection prophylaxis with the monoclonal antibody palivizumab (Synagis) is indicated for all T-cell–deficient infants for the first several years of life (American Academy of Pediatrics, 2012) in the US. During influenza epidemics, anti-influenza medications should be considered, using one of the four licensed anti-influenza virus drugs (amantidine, rimantidine, oseltamivir, or zanamivir, the latter requiring inhalation and recommended only for children 5 years or older) (American Academy of Pediatrics, 2012b).

Immunoglobulin Therapy: All patients with SCID require prophylactic treatment with IVIG prior to stem cell transplantation because their B cells, if present, will either be nonfunctional or lack costimulation by T cells. Even if the T-cell defect is relatively mild (as in some patients with X-linked hyper-IgM syndrome), the patient may have severely impaired B-cell function and should be evaluated for abnormal antibody production. IVIG therapy is indicated for all patients in whom antibody function is deficient (Table 59.3). The dose of IVIG is similar to that discussed earlier for antibody deficiency.

VZIG and/or acyclovir following chickenpox exposure is indicated to prevent generalized chickenpox.

IL-2 cytokines: Patients with T-cell defects have been treated with IL-2 and appear to have achieved some improvement of in vitro T-cell function (Flomenberg et al., 1983).

A few patients with phenotypic SCID were reported to have a defect of IL-2 synthesis with intact IL-2 receptors. IL-2 therapy resulted in temporary restoration of some cellular immunity in a patient with defective IL-2 synthesis (Pahwa et al., 1989).

Cunningham-Rundles et al., (1999a) used PEG-IL-2 in a single patient with idiopathic CD4 lymphopenia and attained a transient favorable clinical and laboratory response.

Other cellular immunodeficiencies in which IL-2 has been used include NEMO syndrome, WAS, DiGeorge syndrome, ataxia-telangiectasia, combined immunodeficiency, and idiopathic CD4 lymphopenia (Roy-Ghanta and Orange, 2010).

G-CSF: G-CSF is of value in T-cell deficiencies when there is an associated neutropenia (e.g., X-linked hyper-IgM syndrome). It is also used in patients who have recently undergone stem cell transplantation to hasten granulocyte recovery. Finally, G-CSF is used to mobilize CD34+ hematopoietic stem cells in donors of peripheral blood stem cells prior to cell harvesting, when used for cellular reconstitution.

Interferons: IFN-γ is of unproven efficacy in most T-cell immunodeficiencies. It has some theoretical value in the treatment of Griscelli syndrome, natural killer (NK) cell deficiency, and bare lymphocyte major histocompatibility complex (MHC) class I deficiency in that it may replace deficient production, enhance NK cytotoxicity, and increase expression of HLA class I antigens.

High-dose IFN-γ therapy is of benefit in the treatment of patients with a partial IFN-γ receptor deficiency or in patients with IL-12 or IL-12 receptor defects (Holland, 2000; Jouanguy et al., 1997).

IFN-α and IFN-γ have been used to treat refractory viral infections such as hepatitis C and IFN-α is also used with success in acute HCV infection (Chapel et al., 2001). Recently IFN-alpha was successful in clearing norovirus in one of two patients with CVID enteropathy (personal communication from Dr. D. Kumararatne).

Other Medical Therapies: Medium-chain triglycerides: In patients with lymphopenia due to gastrointestinal lymphocyte

loss as part of severe protein-losing enteropathy, or in lymphangiectasia of the gut not amenable to medical or surgical treatment, oral medium-chain triglycerides may be of value.

Hormones: Endocrine abnormalities may be associated with T-cell deficiency, notably hypoparathyroidism and hypothyroidism in DiGeorge anomaly and chronic mucocutaneous candidiasis, and hypothyroidism and hypoadrenalism in ataxia-telangiectasia and chronic mucocutaneous candidiasis. These should be treated appropriately, although correction of the endocrinopathies will not affect the T-cell deficiency. Hypothyroidism is also common in CVID patients.

Biotin: In the rare condition of multiple carboxylase deficiency, often associated with candidiasis and severe viral and bacterial infections, the coenzyme biotin given orally at a dose of 1 to 40 mg/day has been curative (Kien et al., 1981)

Folate: Recent description of reversible folate deficiency secondary to a mutation of the proton-coupled folate transporter in a patient with a SCID-like phenotype is encouraging that similar patients may be found in specific transporter proteins (Borzutzky et al., 2009).

Immune lymphocyte infusions: Cytotoxic T lymphocytes specific for CMV from an unrelated donor have been used to combat CMV infection (Horn et al., 2009).

Immune Reconstitution: Restoration of immunity, short of transplantation, is rarely feasible in T-cell immunodeficiencies. Immunomodulating agents such as thymic hormones, levamisole, and transfer factor have not been of proven benefit.

Thymic transplants: Cultured postnatal thymic transplants have been used successfully in infants with DiGeorge syndrome by Markert et al. (2007). Thymic tissue is removed during cardiac surgery from healthy infants, cultured ex vivo to remove mature T cells, and then implanted in the quadriceps muscle. This tissue provides factors that allow maturation of the patient's own T-cell system An alternative procedure for complete DiGeorge syndrome patients with an HLA-matched sibling is stem cell transplantation, which provides mature, long-lasting memory T cells (Land et al., 2007)

PEG-ADA: Hershfield et al. (1995, 1998) reported that 108 patients with the ADA deficiency form of SCID received PEG-ADA, including many of those who have undergone gene therapy (Blaese et al., 1995; Bordignon et al., 1995). Among these patients, 71 remain on therapy. Among those started on PEG-ADA before age 12 months, survival is 74 percent (Hershfield et al., 2003).

The usual dose of PEG-ADA is 30 U/kg given once or twice weekly, with monitoring of plasma ADA levels and erythrocyte adenosine deoxyribonucleotides (dAXP) to assess their response. A few patients have developed an antibody to PEG-ADA with enzyme resistance; this was reversed by increasing the dose of enzyme and treating with corticosteroids and high-dose IVIG (Chaffee et al., 1992).

Clinical improvement is striking in many patients, some of whom have been observed for over 8.5 years, with restoration of protective antibody responses, enhanced proliferative response to injected antigens, and discontinuation of IVIG infusion. PEG-ADA is of particular value for patients too sick to undergo transplantation or those who have no HLA-identical bone marrow donor. Long-term follow-up of these patients shows a gradual decrease in lymphocytes and their proliferative responses, but they are still sufficient to provide protective immunity (Chan et al., 2005) Unfortunately the cost is extremely high (over US $100,000/year).

Attempts to treat purine nucleoside phosphorylase (PNP) and ADA deficiency with various chemicals to block the formation of toxic metabolites or circumvent the biochemical defect have not been successful.

Stem cell transplantation and gene therapy: The treatment of choice, since it may be curative, is HSCT from a matched or partially matched donor using bone marrow, peripheral blood, or cord blood as the source of stem cells. More recently, gene therapy has been successfully performed in a few patients. These procedures are discussed in Chapters 60 and 61.

PHAGOCYTIC IMMUNODEFICIENCIES

General Care

Hygienic precautions: Patients with phagocytic immunodeficiencies, including severe neutropenia (Chapter 51), CGD (Chapter 52), leukocyte adhesion defects (LAD I, II, and III) (Chapter 53), and hyper-IgE syndromes (Chapter 38), are particularly susceptible to cutaneous soft-tissue infections. Thus special care is indicated to avoid cuts, trauma, and environmental situations that lead to exposure to pathogenic organisms (Table 59.1).

Antimicrobials: Most patients require prophylactic antimicrobial therapy. TMP-SMX is usually used, unless neutropenia is present, in which case clarithromycin or ciprofloxacin can be used. Most patients with hyper-IgE syndromes need chronic anti-staphylococcal treatment, such as oral cloxacillin or dicloxacillin, and antifungal therapy, such as fluconazole or itraconazole. Those with an associated antibody deficiency improve on IG therapy. Severe cutaneous infections in these patients are treated with intravenous and/or local antibiotics, often in combination with local steroid therapy.

Neutropenic Syndromes

Patients with neutropenia or defects of cell adhesion and movement are particularly susceptible to periodontitis and oral ulcerations. Good oral hygiene must be stressed, and an antibacterial mouthwash (chlorhexidene) should be used on a regular basis. Prophylactic antifungal drugs should be given if thrush or esophageal candidiasis has occurred.

Congenital neutropenias or neutropenias associated with other immunodeficiency syndromes are usually treated with G-CSF. A persistent absolute neutrophil count less than 200 cells/uL is an indication for its use, particularly if there is an associated infection.

G-CSF and GM-CSF: G-CSF is indicted and approved for severe congenital neutropenia (Dale et al., 1993) (Chapter 51). It has also been used in cyclic neutropenia (Hammond, 1989) WHIM syndrome (Hord et al., 1997) (Chapter 40), and the neutropenias of the hyper-IgM syndrome (Ostenstad et al., 1997) (Chapter 26), XLA (Jacobs et al., 2008), and glycogen storage disease type 1b (Visser et al., 2002).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used in neutropenic syndromes, but the increased cytotoxicity has limited its use (Roy-Ghanta & Jordan, 2010).

Neither cytokine cures these disorders, but they will increase the absolute neutrophil count to near-normal levels. Cure of some of these diseases can sometimes be accomplished by stem cell transplantation

Chronic Granulomatous Disease

General: CGD patients (Chapter 52) are unusually susceptible to Aspergillus and other fungal infections, so patients should rigorously avoid situations where fungal spores can be inhaled or introduced into the skin. Thus, gardening, hay rides, lawn mowing, and digging should be discouraged.

Antimicrobials: CGD patients require continuous prophylactic antibiotics, usually using TMP-SMX (5 mg/kg/day) (Margolis et al., 1990), and antifungal prophylaxis, usually with itraconazole (100–200 mg/day) (Mouy et al., 1994).

Prompt recognition and treatment of infections is of crucial importance. In refractory patients not responding to intravenous antibiotics, leukocyte transfusions may be of value (Yomtovian et al., 1981). Surgical therapy is required for hepatic abscesses or osteomyelitis. Steroids combined with antibiotics and antifungals have also been used in severe refractory infections including inoperable liver abscesses (Yamazaki-Nakashimada et al., 2006, Leiding et al, 2012).

IFN-γ: Immunomodulatory therapy with IFN-γ is used in many centers throughout the United States though less so in Europe. A randomized, double-blind study showed that recombinant human IFN-γ reduced the frequency and severity of infectious episodes in CGD patients, especially those not compliant with antimicrobial prophylaxis (International Chronic Granulomatous Disease Cooperative Study Group, 1991). Since IFN-γ does not increase NADPH oxidative responses, bacterial killing activity or cytochrome b558 levels, it may work by enhancing non-oxidase defenses (Klebanoff et al., 1992; Malmvall and Follin, 1993).

The minimal toxicity and significant clinical effectiveness have led to the recommendation to treat CGD patients of all inheritance patterns in the United States with IFN-γ, 50 μg/M2 body surface, administered subcutaneously three times each week.

However, in Europe, prophylaxis with TMP-SMX and antifungals remains the cornerstone of therapy in adults and older boys; in younger children, BMT is the therapy of choice.

Gastrointestinal and genitourinary complications: CGD patients develop obstructive granulomatous lesions of the esophagus, gastric outlet, and urinary tract. Corticosteroids usually reverse these lesions (Chin et al., 1987). Multiple short courses of high-dose oral steroids may be necessary. Some patients who develop inflammatory colitis are also treated with steroids and/or anti-TNF treatments such as etanercept.

Transplantation: BMT with pretransplant conditioning has been successful in CGD patients, particularly younger patients (Seger et al., 2002; Soncini et al., 2009). Less toxic conditioning regimens prior to transplantation have also been used with promising results (Gungor et al., 2005; Horwitz et al., 2001).

Leukocyte Adhesion Defects

Severe mouth ulcers and periodontal disease are observed in most patients (Chapter 53), so all forms of LAD necessitate good oral hygiene. Continuous antimicrobial therapy may be necessary to prevent cutaneous and deep-seated infections. Daily white blood cell transfusions for 1 or more weeks may be needed to treat severe, life-threatening infections.

Because LAD II is associated with a defect in fucosylation, a patient with a deficiency of the leukocyte selectin CD15a has been treated successfully with oral fucose (Marquardt et al., 1999).

De Ugarte et al., (2002) used GM-CSF formulated for local application to treat nonhealing wounds in patients with LAD and other granulocyte abnormalities (e.g., CGD, glycogen storage disease type 1b).

BMT is readily accomplished in LAD I. Patients with this disorder engraft well because the adhesion molecules that LAD I patients lack contribute to graft rejection (Le Deist et al., 1989).

Hyper-IgE Syndromes

These patients have multiple problems, including skin infections, eczema, osteoporosis, dental abnormalities, and infectious complications such as deep-seated abscesses and pneumatoceles (Chapter 38).

Continuous antimicrobial therapy is usually necessary to keep these patients' cutaneous and deep-seated abscesses under control (Buckley, 2004). Prompt treatment of infections, notably sinusitis and bronchitis, and in many cases continuous prophylactic treatment with antibiotics may prevent acute pneumonia, lung abscesses, and pneumatocele formation. Patients with hyper-IgE syndromes may need prophylactic antifungal therapy since Aspergillus is often the cause of death.

No specific immunotherapeutic regimen has been successful (Freeman and Holland, 2008; Roberts and Stiehm, 1996). IVIG is of minimal benefit but may reduce the IgE levels (Kimata et al., 1995; Wakim et al., 1998). Transfer factor, cimetidine, and ascorbic acid have been used without clinical benefit. Levamisole was inferior to placebo in a controlled, double-blind study (Donabedian et al., 1982). Isotretonin was used in a single case without proven benefit.

Glycogen Storage Type 1b

These patients have neutropenia, functional defects of neutrophil function, and recurrent infections, including sinusitis, pneumonia, and septicemia. Continuous therapy with TMP-SMX has been recommended, and GM-CSF or G-CSF may be beneficial (Hurst et al., 1993).

Chediak-Higashi Syndrome

Although vitamin C corrects some of the defects in vitro, its use in patients with Chediak-Higashi syndrome (Chapter 54) has not been effective (Gallin et al., 1979). Other treatments are supportive, including antibiotics for infection and corticosteroids to treat the accelerated phase of the disease.

BMT has been successful in these patients, particularly if done early, although it will not reverse the neurological abnormalities (Eapen et al., 2007).

Syndromic Mucocutaneous Candidiasis

All patients with syndromic mucocutaneous candidiasis [CMC] (Puel et al., 2012) (Chapter 22) require prophylaxis with anti-fungals, with careful monitoring to avoid resistance to the drug. With increasing understanding of the immunopathogenesis, new therapeutic agents will become available in the next few years.

COMPLEMENT DEFICIENCIES

No specific treatment is available for most complement deficiencies (Sullivan and Winkelstein, 2004). Careful management, however, can improve the health and survival of patients (see Chapter 55). Infections in complement-deficient patients, notably those with C2, C3, C5, C6, C7, C8, properdin, factor H, or factor I deficiencies, should be treated promptly with antibiotics at the first onset of fever or meningismus. An antipneumococcal drug has to be included as this is a common organism, along with meningococci, in these patients.

Long-term penicillin therapy should be considered when meningococcal disease is endemic and for patients with a history of previous severe infections (Potter et al., 1990).

The patient and close household contacts should be immunized against *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitides*. Repeat vaccinations (every 3–5 years) may be necessary if titers diminish to nonprotective levels.

The pediatric conjugated pneumococcal vaccine and the conjugated meningococcal vaccine are recommended in addition to the polysaccharide vaccine for more durable immunity against these illnesses. For unimmunized patients over age 24 months, two doses of conjugated vaccine 8 weeks apart followed by antibody titers 1 month later is recommended.

A deficiency of an early-acting component predisposes to immune complex diseases. Carriers of C2 deficiency may also have a propensity to develop autoimmune diseases. Other family members should be studied for complement deficiency, and if present, genetic counseling can be offered.

Hereditary Angioedema

An exception to the rule that there are no specific therapies for complement deficiencies is hereditary angioedema (HAE). This is an autosomal recessive disorder associated with deficiency or abnormal function of C1 inhibitor (C1INH). The therapy of HAE involves the treatment of acute attacks and prophylaxis of recurrent attacks (Buyantseva et al 2012, Zuraw, 2008).

Management of acute episodes of angioedema must be treated as early as possible and tailored to the extent of the problem. Laryngeal swelling requires urgent evaluation of the airway for possible intubation or tracheostomy, followed by C1INH concentrate (plasma derived or recombinant), given intravenously; this is usually effective within 30 to 60 minutes. Alternatively newly licensed contact system modulators, acting on the kinnin pathway, are also safe and effective (Buyantseva, LV., Sardana, N., Craig, TJ. 2012 Update on treatment of hereditary angioedema Asian Pac J Allergy Immunol 30:89–98). Acute abdominal pain or severe cutaneous swelling can also be treated with C1INH concentrate or kinin modulators.

Less severe attacks can be treated with tranexemic acid, an antifibrinolytic agent. Steroids and antihistamines are no value. Patients with serious symptoms have to be carefully monitored in a hospital to ensure that the angioedema does not progress.

Patients with a history of laryngeal obstruction or serious gastrointestinal involvement are candidates for long-term prevention of attacks. Impeded androgens such as danazol, stanozolol, and oxandrolone are frequently used to increase serum concentrations of the normal C1INH by increasing transcription of the wild-type C1INH gene. The goal of this prophylactic therapy is to maintain the C1INH functional level at approximately 50 percent of normal. Long-term therapy in adults is possible, but patients have to be monitored for adverse reactions, including abnormal liver function tests, microscopic hematuria, and weight gain. Attenuated androgens should not be used in children with open epiphyses on a long-term basis.

For patients with recurrent attacks who fail to respond to or cannot receive androgens, regular infusions of C1INH can be considered for prophylaxis during high-risk periods, and patients are taught to self-infuse in some centers. However, intermittent treatment is usually acceptable.

SUMMARY

General care of the immunodeficient patient requires close monitoring of growth, nutrition, personal hygiene and habits, living conditions, school and work situations, and psychological adjustment to the disease. The most important aspect of the care of an immunodeficient patient is to prevent, recognize, and treat all infectious episodes vigorously. Associated disorders such as anemia, thrombocytopenia, respiratory or gastrointestinal problems, autoimmune and inflammatory disorders are managed by conventional therapy. Avoidance of live-virus vaccines, nonirradiated blood, and CMV antibody–positive blood is crucial for living with some of these illnesses. PCP prophylaxis is necessary in T-cell immunodeficiencies.

Specific therapies that are effective include Ig replacement therapy in patients with antibody deficiency, PEG-ADA injections for ADA deficiency, and IFN-γ for CGD (in some centers) and for IL-12 or IFN-γ receptor deficiencies. Other cytokines, including G-CSF and GM-CSF, are of value in certain selected disorders and replacement C1INH or kinin modulators in HAE.

Stem cell transplantation was pioneered in patients with primary immunodeficiency and continues to be a life-saving procedure for patients with many of these disorders. Minimal

conditioning ("mini-transplants") is being increasingly performed in selected syndromes as a safe alternative to BMT and full conditioning.

Progress in our understanding of the molecular basis of these disorders has paved the way for gene therapy, which is promising and at the same time a challenge.

USEFUL WEBSITES AND SUPPORT GROUPS

Ataxia-Telangiectasia Children's Project, 6685 S. Military Trail, Deerfield Beach, FL 33442; www.atcp.org.

International Patient Organisation for Primary Immunodeficiencies; www. ipopi.org

Jeffrey Modell Foundation, 43 W. 47th Street, New York, NY 10036; http://www.jmfworld.com/

National Immune Deficiency Foundation, 25 W. Chesapeake Avenue, Suite 206, Towson, MD 21204; http://www.primaryimmune.org/

National Organization for Rare Disorders, PO Box 8922, New Fairfield, CT 06812–8923; www.rarediseases.org

PID-UK for Primary Immune Deficiencies – United Kingdom, 199A Victoria Street, London SW1E 5NE; www.piduk.org

Wiskott-Aldrich Syndrome (WAS) Foundation; research@wiskott.org

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60.

BONE MARROW TRANSPLANTATION FOR PRIMARY IMMUNODEFICIENCY DISEASES

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HISTORY OF BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) was first attempted as a treatment for aplastic anemia. In 1939, Osgood et al. infused a few milliliters of marrow into patients with aplastic anemia without benefit (Osgood, 1939). Except in identical twins (Pillow et al., 1966), however, the majority of the early attempts at marrow grafting in man failed, due to lack of knowledge regarding tissue typing, immunosuppression, and supportive care. A major breakthrough was made in the late 1960s when the human major histocompatibility complex (MHC) was discovered (Amos & Bach, 1968). Shortly after that, transplants of unfractionated human leukocyte antigen (HLA)-identical allogeneic bone marrow restored the immune function in two patients with fatal primary immunodeficiency diseases, one patient with severe combined immunodeficiency (SCID) (Gatti et al., 1968) and the other with Wiskott-Aldrich syndrome (WAS) (Bach et al., 1968). The correction of these two conditions, as well as the later correction of many other forms of immunodeficiency by BMT (Antoine et al., 2003; Buckley et al., 1999; Friedrich, 1996), implied that the underlying defects in most immunodeficiencies are intrinsic to cells of one or more hematopoietic lineages and that they are not due to failure of the microenvironment to support the growth and development of those cells.

PRINCIPLE OF BMT

In the early 1950s it was observed that the hematopoietic systems of lethally irradiated mice could be reconstituted by bone marrow cells from a mouse of the same strain (Lorenz et al., 1952). These observations constituted the proof of principle that self-replicating cells in normal bone marrow can give rise to erythrocytes, granulocytes, cells of the monocyte-macrophage lineage, megakaryocytes, and immunocompetent T and B cells (Brenner et al., 1993; Wu et al., 1967, 1968). Based on these findings, BMT has been used in attempts to replace defective, absent, or malignant cells of the recipient with normal replicating hematopoietic and immunocompetent cells, first in children with therapy-resistant leukemia using an identical twin as donor after supralethal irradiation (Thomas, 1957, 1959). However, allogeneic marrow grafting continued to be mainly unsuccessful. Between 1939 and 1969, 203 transplants were reported, predominately in patients with aplastic anemia, leukemia, and malignancy, but also in 15 patients with immunodeficiencies (Bortin, 1970). The only three surviving patients were those treated for immunodeficiency, including the two mentioned above. The causes of death were mainly graft rejection, infection, and graft-versus-host disease (GVHD).

Unfractionated BMT offers little risk to the donor, as it involves removal of a tissue that is readily regenerated. Bone marrow cells are usually obtained by aspiration from multiple sites along the iliac crests, the sternum, or (in small children) from the upper third of the tibia, while the donor is under general anesthesia (Martin et al., 1987; Thomas et al., 1975). The aspirate is placed in heparinized tissue culture medium and then passed through metal screens to remove bone spicules. If T-cell depletion is necessary, the marrow is first processed to remove T cells; nucleated marrow cells are then enumerated and the cells are given intravenously to the recipient.

HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR PRIMARY IMMUNODEFICIENCY DISEASES: SPECIAL CONSIDERATIONS

Primary goal: immune reconstitution. Unlike the situation in patients with malignancies or global bone marrow

failure, the goal of hematopoietic stem cell transplantation (HSCT) in patients with primary immunodeficiency is to provide normal hematopoietic donor stem cells to correct the underlying genetic deficits in the recipient's immune system. In classic severe combined immunodeficiency (SCID), no pre-transplant immunosuppression is needed because these patients lack T cells and are therefore unable to reject a graft (Buckley, 2004). In other forms of primary immunodeficiency or hypomorphic ("leaky") SCIDs, however, there is usually enough residual T-cell function to cause rejection. Therefore, pre-transplant conditioning agents must be used. Drugs commonly used to ensure the acceptance of solid organ grafts have deleterious effects on the very cells one is trying to engraft in patients with genetically determined immunodeficiency. Thus, any chemotherapy must be given *prior* to infusion of the hematopoietic stem cells to avoid injury to the donor cells.

GVHD: A MAJOR OBSTACLE TO SUCCESSFUL BMT

In further contrast to the situation in solid organ transplantation, successful marrow engraftment in the early days of transplantation required HLA identity (at least for HLA class II determinants) between donor and recipient, primarily because of the extreme susceptibility of the HSCT recipients to GVHD. In the past, this was particularly problematic for patients with SCID and complete DiGeorge syndrome because they cannot reject foreign T cells and therefore developed severe, sometimes fatal, GVHD caused by T cells in unirradiated blood products even before transplantation. Until 3 decades ago, the requirement for an HLA-identical donor limited the possibility of a BMT to patients with an HLA-identical sibling donor, and the likelihood that any given sibling would be a match is only one in four. However, one of the most important advances in the treatment of primary immunodeficiency diseases over the past decades has been the development of methods to avoid GVHD by T-cell depletion and thus to enable transplantation of half-matched (haploidentical) parental stem cells (Antoine et al., 2003; Buckley, 2004; Buckley, 2011; Buckley et al., 1999; Friedrich, 2010).

THE CHALLENGE OF ONGOING INFECTIONS

Although greater awareness of primary immunodeficiencies among pediatricians has led to earlier diagnosis of these rare conditions in recent years, infants and children with primary immunodeficiency often become infected with opportunistic infectious agents prior to diagnosis. These infections cause particular problems from the standpoint of urgency of need for transplantation and may limit the possibility of using chemotherapeutic conditioning agents. This is particularly true for infections with viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenoviruses, and parainfluenza viruses, for which limited or no effective treatment is available (Buckley, 2004; Teigland et al., 2013). Important progress has been made in the past decade, as molecular diagnosis is now readily available. Detection of increasing viral load, even prior to the onset of clinical symptoms, enables the physician to initiate preemptive antiviral treatment to avoid organ damage and to monitor the efficacy of antiviral treatment strategies (Watzinger, 2004). However, most patients with SCID are not discovered prior to their developing one of these viral infections and they can have a fulminating course in which the anti-virals are ineffective. Toxicity of different antiviral agents is also a major concern, as well as development of resistance to therapy. This is especially true for patients with haploidentical donors, in whom the delay for efficient T-cell reconstitution after HSCT necessitates often prolonged antiviral treatment, as sufficient host T-cell response is essential to definitively clear the virus. Therefore, the development of adoptive immunotherapy based on the infusion of virus-specific donor T lymphocytes is currently receiving major attention. This therapeutic strategy is promising in viral infections with EBV (Heslop, 2010), CMV (Feuchtinger, 2010), or adenovirus (Feuchtinger, 2008) but may increase the risk of GVHD. Adoptive immunotherapy for invasive aspergillosis is also under development (Tramsen, 2009).

PERSISTENCE OF TRANSPLACENTALLY ACQUIRED MATERNAL T CELLS

Leukocytes from the mother often enter the fetal circulation during pregnancy. SCID fetuses with transplacentally acquired maternal T cells have no capacity to reject these cells, which frequently persist in the infant's circulation. These maternal T cells are usually unable to respond normally to mitogens and fail to correct the infant's underlying immunodeficiency. They may or may not cause clinical signs of GVHD, such as "eczema" or splenomegaly (Palmer et al., 2007). They can, however, create special problems (or benefits) when BMT is performed for these SCID infants. If a donor other than the mother is used and no pretransplant chemotherapeutic conditioning is given, there is always the risk of a graft-versus-graft (GVG) reaction. If unfractionated HLA-identical sibling marrow is infused, this can lead to rejection of the maternal T cells, with or without a clinical GVG reaction (Friedman et al., 1991). By contrast, if a T-cell–depleted haploidentical graft is given from the father without pretransplant conditioning, rejection of the paternal graft can occur, again with or without a clinical GVG reaction (Palmer et al., 2007). On the other hand, if T-cell–depleted haploidentical stem cells are given from the mother without pretransplant conditioning, immune reconstitution is often accelerated (Barrett et al., 1988), again with or without clinical signs of GVHD. However, if GVHD occurs in this setting, it usually requires either no treatment or only transient use of steroids.

CONDITIONING REGIMENS ARE NOT ALWAYS NEEDED

In contrast to the situation in patients with malignancy or marrow aplasia, it has been repeatedly shown that infants

with SCID or with complete DiGeorge syndrome who have absent T-cell function do not require pretransplant immunosuppression for successful BMT (Buckley, 2004, 2011; Buckley et al., 1999; Myers et al., 2002). Opinions differ on whether SCID patients should be given conditioning, and some transplant centers have recommended conditioning for SCID patients with high numbers of NK cells (Antoine et al., 2003). However, studies of NK cell KIR receptors in SCID patients and their donors did not reveal any significant effect of these receptors on graft acceptance or GVHD (Keller et al, 2007). Patients with most non-SCID types of primary immunodeficiency or hypomorphic SCID, especially those with significant residual T-cell numbers and function, need to be preconditioned with chemotherapeutic agents to prevent graft rejection (see below) (Parkman, 1991).

POSTTRANSPLANT GVHD PROPHYLAXIS IS NOT ALWAYS NEEDED

As is the case for pretransplant conditioning, posttransplantation prophylaxis against GVHD is not necessary in SCID patients after transplantation of HLA-identical or rigorously T-cell–depleted HLA-haploidentical marrow (Buckley, 2011; Buckley et al., 1999; Myers et al. 2002). In non-SCID patients, GVHD prophylaxis is recommended in a geno-identical or pheno-identical transplant setting, but it can be omitted in T-cell–depleted haploidentical transplants. Rigorous depletion of donor T cells is the most effective means of GVHD prophylaxis.

SOURCES OF STEM CELLS FOR HSCT AND DONOR SELECTION

For patients with SCID and many other forms of primary immunodeficiency, allogeneic HSCT is currently the only curative approach. (Antoine et al., 2003; Buckley, 2004, 2011; Friedrich, 1996; Haddad et al., 1998; Gennery et al., 2010; Neven, 2009). Transplantation by using a geno-identical sibling donor results in highest survival and is the treatment of choice, but only a few patients have an HLA-matched sibling. The various sources of stem cells are listed in Table 60.1.

Table 60.1 **SOURCES OF STEM CELLS FOR TRANSPLANTATION**

HLA-identical sibling marrow
Haploidentical parental marrow
T-cell–depleted by soy lectin/sheep red blood cells
T-cell–depleted by monoclonal antibodies
T-cell–depleted by CD34/CD133 positive selection
Matched unrelated adult marrow
Unfractionated
T-cell–depleted by CD34/CD133 positive selection
Unfractionated related or unrelated cord blood

UNFRACTIONATED GENO-IDENTICAL BONE MARROW OR PERIPHERAL BLOOD

If a patient with SCID or other form of primary immunodeficiency is fortunate enough to have an HLA-identical sibling, unfractionated bone marrow cells can be given, and this is the treatment of choice. In most cases, both T-cell and B-cell immunity have been fully reconstituted by such matched transplants, with evidence of function detected as early as 2 weeks following transplantation due to adoptive transfer of mature donor T, B and NK lymphocytes. Patients with SCID have required far fewer unfractionated nucleated marrow cells for successful immune reconstitution than the number needed to treat leukemia or aplastic patients; as few as 4×10^6 $CD34^+$ cells per kilogram recipient body weight have resulted in immunological reconstitution (Friedrich, 1996). However, there is some evidence that higher numbers of stem cells result in better immune reconstitution (Buckley et al., 1999). Unfractionated marrow cells will also engraft in HLA-disparate SCID recipients who lack cellular immunity, but fatal GVHD can be anticipated in the haploidentical setting because unfractionated marrow cell suspensions contain, in addition to stem cells, mature T cells. In rare cases, umbilical cord blood from an HLA-identical sibling may be available.

MATCHED UNRELATED BONE MARROW, PERIPHERAL BLOOD, OR CORD BLOOD

In the absence of a geno-identical donor, matched unrelated donors may be an alternative stem cell source. Donor registries were created to facilitate volunteer stem cell donor searches. In 1988, the Bone Marrow Donors Worldwide (BMDW) was created as an initiative of the Immunobiology Working Party of the European Group of Blood and Marrow Transplantation (EBMT). In 1992, the National Marrow Donor Program was established in the United States. Since then, numerous similar registries have been created. BMDW's first edition in February 1989 contained the donor files of eight registries with a total of 155,000 voluntary donors. In its last edition, 64 stem cell donor registries from 44 countries and 44 cord blood banks from 26 countries are listed in the BMDW directory, including currently almost 15 million donors: more than 14.4 million voluntary stem cell donors as well as more than 400,000 cord blood units (source: http://www.bmdw.org).

Despite the growing number of unrelated volunteer stem cell donors, the probability of identifying a matched unrelated donor ranges from about 60 to 70 percent for Caucasians to under 10 percent for some ethnic minorities, depending on the patient's genetic background and the diversity of HLA within his or her ethnic population (Beatty, 1995; Koh, 2008). It is thus still difficult to identify an unrelated donor for patients with rare HLA antigens, and only approximately 30 percent of patients will have an HLA-matched sibling, a matched unrelated stem cell donor, or a matched unrelated cord blood unit. Furthermore, the search for an unrelated donor is a cumbersome and time-consuming process that necessitates identifying, typing, and harvesting cells from the unrelated donor.

As the average time interval between initiation of the search and the donation of marrow is about 4 months (Heemskerk, 2005), this approach is not practical for patients who urgently need transplantation. This option is also quite expensive (Tiercy et al., 2000).

T-CELL–DEPLETED HAPLOIDENTICAL BONE MARROW OR PERIPHERAL BLOOD.

For patients without any possibility of identifying an HLA-matched related donor, or for patients in such a poor clinical condition that the time needed for the unrelated donor search is too long, an alternative approach is the use of mismatched related family donors. As most of these donors will share only one HLA haplotype with the recipient, they are referred to as haploidentical donors. The immense advantage of parental haploidentical donors is due to the fact that they are not only rapidly available for the transplantation but that they remain easily accessible during the posttransplantation course for eventual further stem cell harvesting, such as a stem cell boost or adoptive cellular therapy.

In contrast to previous reports (Grunebaum, 2006), it has been recently shown in the so far largest cohort study on the outcome of patients with primary immunodeficiencies that in SCID patients the use of a matched unrelated donor does not confer a significant advantage over a haploidentical donor, whereas in the non-SCID patients matched unrelated donors or other identical relatives still show a clear advantage over haploidentical donors (Gennery et al., 2010). Thus, in SCID patients who lack an HLA-identical family member, the treatment of choice is a T-cell–depleted haploidentical transplant from a family member, unless a matched unrelated donor is available very rapidly and the patient's clinical condition is sufficiently stable to allow a delay in HSCT.

The improvement in use of haploidentical stem cell donors has been made possible through major advances in graft manipulation, (Handgretinger, 2008). Following the leads from his own work in mice (Reisner et al., 1978) and from Muller-Ruchholtz et al.'s (1976) work in rats, Reisner et al. (1983) demonstrated in the early 1980s that removal of most mature T cells from half-matched parental marrow allowed successful immune reconstitution of human infants with SCID by the remaining cells without subsequent GVHD. Since that time, this principle has successfully been applied many times to the treatment of SCID (but not as successfully to other primary immunodeficiency diseases) (Table 60.2; adapted from Gennery et al., 2010) (Antoine et al., 2003; ; Buckley, 2011; Buckley et al., 1986, 1999; Dror et al., 1993; Fischer et al., 1986; Giri et al., 1994; Moen et al., 1987; O'Reilly et al., 1986; Stephan et al., 1993; Wijnaendts et al., 1989).

The option to use a haploidentical donor, however, virtually ensures that all infants with SCID can benefit from HSCT. Usually a mother or father (rather than a sibling) is the donor, since the volume of marrow donation required is much larger for this type of transplant. In SCID patients with persistence of transplacentally transferred maternal T cells, a mother is often a better donor than a father, unless there are health issues that preclude the use of the mother (i.e., if she is pregnant or positive for HIV or hepatitis B antigen). In the remaining patients, the donor choice should take into consideration the number of shared HLA antigens, CMV and EBV positivity, as well as the age and number of preceding pregnancies, which may increase the risk of GVHD (Atkinson, 1986; Loren, 2006).

One condition in which a haploidentical transplant is expected to have no benefit is the complete DiGeorge syndrome, where T-cell–depleted marrow cells will be ineffective to restore T-cell immunity, since there is no thymus to promote the thymus-dependent T-cell differentiation of donor stem cells. The reported successful outcome after unfractionated HLA-identical marrow transplants into complete DiGeorge patients is due to the adoptive transfer of mature T cells that were "educated" in the donor's thymus (Goldsobel et al., 1987).

T-CELL DEPLETION METHODS

High transplant-related mortality, due to early and severe GVHD when using T-cell–replete haploidentical bone marrow, was a major obstacle to overcoming HLA barriers and represented an area of intense research in the 1970s. An important milestone was reached when Reisner et al. developed a technique to efficiently remove mature T cells from the graft prior to HLA-mismatched transplantation to prevent GVHD (Reisner, 1978).

Soybean lectin, sheep erythrocyte agglutination. The first technique successfully used for ex vivo T-cell depletion of the donor marrow involves agglutination of most mature marrow cells with soybean lectin, followed by sedimentation of clumped cells and subsequent removal of T cells from the unagglutinated marrow by sheep erythrocyte rosetting and density-gradient centrifugation (Reisner et al., 1983; Schiff et al., 1987). The final cell preparation contains few if any lymphocytes, is phytohemagglutinin (PHA) and mixed leukocyte culture (MLC) nonresponsive, consists of immature myeloid cells and stem cells, and represents less than 5 to 10 percent of the initial number of nucleated marrow cells (Schiff et al., 1987). This method, unlike CD34 selection devices, leaves all other immature marrow cells (including CD34-negative stem cells) available in the cell suspension for use in the transplant. It allows stable immunological reconstitution in SCID patients after haploidentical parental donor transplants and reduces significantly the incidence of acute and chronic GVHD, as observed first by Reisner et al. (1983) in the early 1980s and confirmed by several other groups in the following years (reviewed in (Friedrich, 2010). Newly developed T cells were found to be tolerant to the HLA-haploidentical recipient.

Monoclonal antibody and complement lysis. Another method of depleting postthymic T cells from donor marrow is incubation with monoclonal antibodies to human T cells plus a source of complement (Filipovich et al.; 1984; Reinherz et al., 1982; Waldmann et al., 1984). Antibodies employed for this complement-mediated T-cell depletion have included T12, Leu 1, CT-2, OKT3, and CD52 (Campath-1). T-cell

Table 60.2 TYPE OF IMMUNODEFICIENCY ACCORDING TO DONOR ORIGIN AND HLA MATCHING

IMMUNODEFICIENCY	*N* (% OF CATEGORY)		RELATED DONOR: GENOTYPICALLY HLA IDENTICAL	RELATED DONOR: PHENOTYPICALLY HLA IDENTICAL	RELATED DONOR: HLA-MISMATCHED	UN-RELATED
SCID: Total	699		135	68	415	81
Reticular dysgenesis	19	(3)	2	1	14	2
ADA deficiency	75	(11)	25	6	31	13
T-B-	206	(29)	39	30	117	20
T-B+	345	(49)	54	24	236	31
Other	54	(8)	15	7	17	15
Non-SCID: Total	783		251	65	243	224
Wiskott-Aldrich Syndrome	168	(21)	48	10	51	59
T cell deficiencies						
Omenn syndrome	65	(8)	13	7	33	12
PNP deficiency	11	(1)	2	3	3	3
HLA class II deficiency	62	(8)	19	10	28	5
CD40 ligand deficiency	36	(5)	10	0	1	25
Other	152	(19)	48	9	50	45
Phagocytic cell disorders						
Agranulocytosis	10	(1)	3	0	2	5
CGD	41	(5)	29	0	0	12
Leukocyte adhesion deficiency	34	(4)	13	2	18	1
Other	7	(1)	4	1	1	1
Hemophagocytic syndromes						
Familial lymphohistiocytosis	99	(13)	28	8	40	23
Chédiak-Higashi syndrome	26	(3)	11	6	3	6
XLP (Purtillo)	19	(2)	3	0	5	11
Griscelli disease	15	(2)	7	3	2	3
Other	38	(5)	13	6	6	13

ADA, adenoside deaminase; PNP, purine nucleoside phosphorylase; SCID, severe combined immunodeficiency; XLP, X-linked lymphoproliferative disease

Adapted from Gennery et al, *J Allergy Clin Immunol* 126(3):602–10, 2010

depletion is not as effective as with the above approach, possibly due to modulation of T-cell antigens from the surface of the T cells without destroying them. As a consequence, more frequent and severe GVHD has been observed.

CD34+ cell selection by affinity columns. The most common method of T-cell depletion is the use of commercially available antibody affinity columns that positively select for $CD34^+$ cells (Gordon et al., 2002; Handgretinger et al., 2001; Schumm et al., 1999). Further development of this technique resulted in an increased purity of the isolated $CD34^+$ cells and a high degree of depletion of T cells (4.5- to 5-log reduction) and B cells (>3-log reduction) (Handgretinger, 2008). However, in this method, CD34-negative stem cells are discarded, along with other cells that could be important in promoting immune reconstitution, such as dendritic cells. This rigorous T-cell depletion requires no additional pharmacological GVHD prophylaxis. A major concern is the delayed immune reconstitution with the genuine risk of severe viral infections, especially CMV and adenovirus infections.

CD133 selection. Recently, the pentaspan molecule CD133 has been judged to be a marker of more primitive hematopoietic progenitors. Lang et al. (2004) evaluated a CD133-based selection method in 10 pediatric patients given stem cells from matched unrelated (n = 2) or mismatched related donors (n = 8). Engraftment occurred in all patients (sustained primary, n = 8; after reconditioning, n = 2). No primary acute GVHD of a magnitude greater than grade II or chronic GVHD was observed.

CD3/CD19 depletion. This innovative graft engineering of peripheral stem cells is based on depletion of $CD3^+$ T cells and $CD19^+$ B cells by negative selection, thus allowing NK cells, monocytes, and dendritic and myeloid cells to remain in the graft (Handgretinger, 2008). This technique, which should yield cell suspensions more comparable to the soy lectin method, has been used so far especially in children

with hematological malignancies; early studies indicate that patients with primary immunodeficiency may also benefit from this method.

Unfractionated, matched unrelated adult marrow. Most patients with primary immunodeficiency will not have an HLA-identical sibling donor, and matched unrelated marrow donors (MUDs) are now widely used, especially in non-SCID patients, who do not require urgent transplantation. As humans are an outbred population, the matching is unlikely to be perfect (Tiercy et al., 2000) for matched unrelated adult bone marrow or unrelated cord blood. Minor locus histocompatibility antigen differences are also much more likely to be present than in the related donor setting, resulting in a higher probability of GVHD. Another important drawback with using MUD adult donors is that it takes approximately 4 months (6 weeks to 12 months) to identify an unrelated adult donor.

Cord blood. One rich source of hematopoietic stem cells for transplantation is unfractionated cord blood (Wagner et al., 1995). Part of the rationale for using cord blood is that the incidence and severity of GVHD appears to be lower with MUD cord blood than with MUD adult unfractionated marrow transplants, even in the presence of one or two HLA antigen mismatches (Barker & Wagner, 2002; Wagner et al., 2002). However, well-matched cord blood units should be preferred, as GVHD contributes to a poorer outcome, especially in immunodeficient patients with preexisting viral infections. The fact that it generally takes less time to locate a matched cord blood unit than an adult MUD may privilege the use of unrelated umbilical cord blood over MUD in patients with immunodeficiencies. The efficacy of unrelated cord blood transplants with regard to immune reconstitution has been documented in several series (Bhattacharya, 2005; Frangoul, 2010). Several groups have also reported successful cord blood transplantation from HLA-identical siblings in patients with genetic disorders, including immunodeficiencies (Barker & Wagner, 2002; Grewal et al., 2003; Stary et al., 1996; Vowels et al., 1993; Wagner et al., 1995, 2002). Affecting the success rate of cord blood transplants is the number of nucleated cells present in the unit, especially the number of $CD34^+$ cells: it has been shown that at least 1.7×10^5 $CD34^+$ cells per kilogram recipient body weight are needed (Wagner et al., 2002). In addition, there may be a much longer period of thrombocytopenia following cord blood transplants than after HLA-identical unfractionated marrow transplants (Thomson et al., 2000).

Most of the current protocols for cord blood transplantation call for pretransplant conditioning, regardless of the underlying genetic diagnosis of the patient. Successful umbilical cord transplantation has, however, been achieved in some SCID patients without pretransplant conditioning (Toren et al., 1999). GVHD prophylaxis is routinely given after transplants from unrelated cord blood donors. The conditioning and GVHD prophylaxis both contribute to a long period of immune impairment posttransplantation, which is self-defeating if the goal of transplantation is immune reconstitution. Control of viral infections may also be a concern, as cord blood lacks viral-specific cytotoxic T cells, and immune reconstitution is slower than following HSCT with replete marrow, resulting in delayed viral clearance. Furthermore, the nonavailability of the original donor for a boost may be another disadvantage of unrelated cord blood transplantation. Ex vivo expansion methods of cord blood progenitor cells prior to stem cell transplantation are under evaluation.

FETAL LIVER CELLS AS A STEM CELL SOURCE

Anecdotal cases have been reported using fetal liver cells to effect immune reconstitution in SCID (Keightley, 1975). Subsequent series of fetal liver cell transplantations in a larger number of patients confirmed the potential of these cells to correct T-cell immunodeficiency, but durable engraftment was achieved in less than 30 percent of the patients, and it can be speculated that (in the absence of conditioning prior to transplantation) the observed donor T lymphocytes derived from committed lymphoid progenitors rather than from engrafted hematopoietic stem cells (Rappeport, 2010).

IN UTERO BMT

The availability of early prenatal diagnosis for several primary immunodeficiencies offers the option of in utero treatment. Two infants with γ_c-deficient SCID received $CD34^+$ paternal stem cells intraperitoneally during the first half of gestation and developed immune function postnatally (Flake et al., 1996; Wengler et al., 1996). Previous experience with in utero treatment had not been very successful (Touraine, 1996). Since then, a number of in utero transplantations for immunodeficiencies have been performed (reviewed in (Muench, 2005). The major concern is that in utero transplantation necessitates invasive procedures on the fetuses, and on more than one occasion. Furthermore, there is always the risk of GVHD or of a GVG reaction because of the likely presence of transplacentally transferred maternal T cells. If either GVG or GVHD occurs, there is no way of detecting or treating it; thus, fetal wastage could be a consequence. Benefits of in utero transplantation over immediately postnatal transplantation in settings where parents do not wish pregnancy termination following prenatal SCID diagnosis have to be carefully assessed. In utero BMT obviously may reduce the risk of infectious complications. On the other hand, early postnatal haploidentical BMT in SCID offers excellent results, as it has been found to be successful in more than 94 percent of cases (Buckley, 2004; Buckley et al., 1999; Myers et al., 2002; Buckley, 2011). This makes it difficult to justify the risk to the fetus associated with in utero transplantation procedures, for which an overall survival rate of only 64 percent has been reported (Muench, 2005). To date, in utero transplantation is thus not generally recommended in families with a known risk for SCID. Immediate diagnosis at birth through newborn screening (Buckley, 2012) and prompt transfer to an experienced center performing stem cell transplantation for immunodeficiency remains the treatment of choice

GRAFT REJECTION AND GVHD

Unlike solid organ transplantation, allogeneic marrow transplantation is complicated not only by the possibility of graft rejection by the recipient, but also by the potential of immune cells in the transplanted marrow to react against the recipient (i.e., causing GVHD).

Prevention of graft rejection. As noted above, immunosuppressive and myeloablative agents used to prevent graft rejection must be given *prior* to the infusion of marrow cells to avoid injury to the donor cells. In transplant recipients with some potential to reject grafts, factors influencing the likelihood of engraftment of donor marrow cells are (1) the degree of immunocompetence of the recipient, (2) the degree of MHC disparity between donor and recipient, (3) the degree of presensitization of the recipient to the histocompatibility antigens of the donor, (4) the number of stem cells administered, (5) the type of conditioning regimen given the recipient, and (6) whether or not T-cell–depletion techniques are used. Initially, total body irradiation (TBI) was used to prepare non-SCID immunodeficiency patients for BMT, but it was later recognized that TBI could be safely replaced by busulfan at dosages (16–20 mg/kg total) that did not produce comparable adverse late effects (Blazar et al., 1985). This is due to the fact that preparation of the recipient needs only be directed at immunosuppression and "spacing" (i.e., making room in the marrow for donor cells). Concerns were the reduced bioavailability of the oral busulfan in young patients (below the age of 2 to 4 years) (Blazar et al., 1985; Vassal et al., 1993) and the observation of a high variability of busulfan disposition among young children (by a factor of 6) (Vassal et al., 1993). These results demonstrate the need for an individual, close adjustment of busulfan in infants and young children. A major achievement was the introduction of an intravenous form of busulfan and the development of busulfan plasma-level monitoring and dose adjustment at the individual level (Bartelink, 2009; Vassal, 2008). The drug can also be given only once or twice a day (Bartelink, 2008; Bostrom et al., 2003; Dalle et al., 2003; Fernandez et al., 2002). Until recently, there was a general consensus on the use of 8 to 16 mg/kg busulfan and 200 mg/kg cyclophosphamide with or without antithymocyte globulin (ATG) as a conditioning regimen in non-SCID immunodeficiency in the setting of HLA-identical BMT (Parkman, 1991). Concerns on the toxicity of this conditioning regimen, especially with regard to the development of veno-occlusive disease (VOD) in small infants, led to the introduction of fludarabine-based regimens in association with busulfan with or without serotherapy (ATG or Campath H1), which have already been used successfully in HSCT for nonmalignant pediatric diseases (Jacobsohn, 2004). This conditioning allows stable engraftment in immunodeficient patients with a geno- or phenol-identical donor, but not in non-SCID patients receiving a T-cell–depleted haploidentical graft (unpublished experience of the group of Alain Fischer). In latter cases, the addition of thiotepa (10–15 mg/kg) allows engraftment without inducing an exaggerated toxicity. So far, the results of the busulfan/fludarabine-based regimen are encouraging, and a decrease in toxicity with regard to VOD has been observed. Other European centers prefer fludarabine/treosulfan-based conditioning (Slatter, 2011).

NONMYELOABLATIVE CONDITIONING REGIMENS

The interest of myeloablative conditioning lies in the prevention of rejection by removing host-versus-graft reactivity, creating marrow space, and if necessary eradicating malignant cells. In this setting, a full chimerism is often achieved in the early posttransplant period. In HSCT for genetic disorders other than SCID, myeloablative doses of cytoreductive agents are used to eradicate genetically abnormal stem cells before allogeneic transplantation. In patients with preexisting organ damage and infections, significant morbidity and mortality may be observed after traditional myeloablative conditioning regimens employing busulfan and cyclophosphamide or irradiation. Therefore, there has been increasing interest in developing conditioning regimens that are less toxic (Barta et al., 2001; Feinstein et al., 2001; Gaspar et al., 2002; Horwitz et al., 2001; Mielcarek et al., 2002; Nagler et al., 1999; Pulsipher & Woolfrey, 2001; Woolfrey et al., 2001;Satwani et al 2008).

Nonmyeloablative regimens may be an attractive alternative for patients with primary immunodeficiencies in whom the eradication of malignancy is not required. Immune deficiency syndromes are examples of disorders that primarily affect a single lineage, such as the lymphoid or myeloid lineages. Therefore, unlike in malignant disease, a complete donor chimerism is not necessary to cure the disease, as reconstitution of an unaffected lineage is not required and for most immunodeficiencies, a mixed chimerism is sufficient to achieve immune reconstitution.

The introduction of the first nonmyeloablative conditioning regimens was based on the hypothesis that the donor T cell itself participates in the creation of its own marrow niche through a subclinical GVH reaction directed toward recipient hematopoietic cells, thus avoiding the need for myeloablative conditioning and improving the immediate and long-term outcome of the patient through less toxicity (Slavin, 1998). Nonmyeloablative conditioning regimens using fludarabine, ATG, and low-dose busulfan (8 mg/kg) have shown that intense immunosuppression can be sufficient to establish full or partial donor chimerism and that full myeloablation is not required for creation of marrow space. They are also referred to as "reduced-intensity conditioning" (RIC), and they produce significantly less toxicity while allowing partial or full chimerism in most patients (Slavin, 1998).

In contrast to these RIC regimens (Slavin, 1998), which still contain myeloablative molecules like busulfan or melphalan but at a reduced dose, "minimal intensity conditioning" (MIC) regimens are based on immunosuppressive agents and are truly nonmyeloablative. As they rely on graft-versus-marrow effect to create marrow space, graft rejection may occur in certain patients; however, graft rejection is less of an obstacle in patients with immunodeficiency. There is also concern that MIC regimens might be associated with an increased GVHD risk (Veys, 2010). The different approaches

of reduced-intensity transplantation have recently been reviewed by Veys (2010); his group was also the first to report the combination of fludarabine, melphalan, and ATG (FMA) in eight patients with immunodeficiencies (SCID and non-SCID patients with significant comorbidities before HSCT), seven of whom were surviving with donor cell engraftment 8 to 17 months after transplant (Amrolia, 2000). Since then, an overall survival of 82 percent was reported in a large series of 113 patients who had undergone nonmyeloablative conditioning between 1998 and 2006 (93 RIC and 20 MIC) (Satwani, 2008). 18 patients presented with an important pretransplant organ toxicity and 81 percent had stable donor engraftment.

Although nonmyeloablative regimens are significantly less cytotoxic than high-dose alkylating agents and TBI, they are profoundly immunosuppressive. Thus, viral infections/reactivations (including CMV, adenovirus, and EBV) remain clinical obstacles when nonmyeloablative stem cell transplants are performed. GVHD prophylaxis with cyclosporine and methotrexate, with added mycophenolate mofetil in some cases, has been necessary because GVHD is common following nonmyeloablative transplantation.

Special attention has to be paid to the choice of the conditioning regimen for radiosensitive SCID patients who present with increased sensitivity to ionizing radiation. In consequence they also display increased sensitivity to alkylator-based chemotherapy, which has been traditionally used prior to HSCT. The development of novel nontoxic conditioning regimens for patients with intrinsic radiosensitivity and chemosensitivity will be a challenge for the future, with a potential benefit not only for radiosensitive SCID patients but also for other immunodeficient patients undergoing HSCT (Dvorak, 2010).

Use of anti-CD52, Campath 1, monoclonal antibody in vivo. It has become common practice to include the in vivo administration of alemtuzumab (anti-CD52, Campath 1) in reduced-intensity or conventional pretransplant ablation protocols for patients who are receiving peripheral blood stem cell transplants. While this approach has been effective in controlling GVHD and improving engraftment, there were concerns about a longer period of immunodeficiency posttransplantation (Chakrabarti et al., 2004) and about the development of lethal lymphoproliferative disease (Snyder et al., 2004). Although alemtuzumab-based T-cell–depleted allogeneic HSCT seems to be associated with a high frequency of EBV reactivation, it has been shown recently that the risk of post-transplant lymphoproliferative disorder is low (<1 percent) when using a strategy of preemptive rituximab therapy (Carpenter, 2010). Patients need to be carefully monitored for infection/reactivation of viruses.

GVHD

GVHD is the major barrier to widespread successful application of BMT for the correction of many diseases (Deeg & Henslee-Downey, 1990; Ferrara, 2009; Ferrara & Deeg, 1991; Glucksberg et al., 1974; Martin et al., 1987; Parkman, 1991; Reddy, 2009).

PATHOPHYSIOLOGY OF GVHD

Acute GVHD reflects an exaggerated but normal inflammatory response of donor T cells to host antigens (Ferrara, 2009), especially the highly polymorphic HLAs, which are encoded by the MHC. Therefore, the incidence of acute GVHD is directly related to the degree of HLA disparity between the donor and the recipient. However, despite HLA identity between patient and donor, approximately 40 percent of recipients of HLA-identical grafts will still develop acute GVHD due to genetic differences in "minor" histocompatibility antigens.

The complex interplay between the donor and host innate and adaptive immune responses leading to GVHD (recently reviewed by Choi, 2010; Ferrara, 2009; and Paczesny, 2010) can be divided in three different phases (Reddy, 2003). In the first phase, antigen-presenting cells (APCs) are activated by the underlying disease, prior infections, and the transplant conditioning regimen, leading to tissue injury in the recipient (Ferrara & Deeg, 1991) with subsequent release of proinflammatory cytokines (Sorror, 2004). Increased levels of TNF-α receptor I, a surrogate marker for TNF-α, 1 week after HSCT, strongly correlated with the later development of GVHD (Choi, 2008). Damage to the gastrointestinal tract from the conditioning may lead to systemic translocation of additional inflammatory molecules that further enhance the activation of host APCs (Hill, 2000). The second step involves donor T-cell activation, proliferation, differentiation, and migration in response to host APCs.

The effector phase of acute GVHD involves both cellular (such as cytotoxic T lymphocytes and NK cells) and soluble inflammatory mediators (such as TNF-α, IFN-γ, IL-1, and nitric oxide) (Ferrara & Deeg, 1991). The complex interplay between these soluble and cellular mediators amplifies local tissue injury and further promotes inflammation and target tissue destruction (Ferrara, 2009).

In contrast to acute GVHD, the pathophysiology of chronic GVHD is poorly understood (Ferrara, 2009), and there are limited data specific to pediatrics (Baird, 2010). Historically, alloreactive donor T cells have been thought to be the primary factor implicated in chronic GVHD. However, the role of direct T-cell–mediated allogeneic immune responses in cGVHD is not clear, and there is no strong correlation between the number of minor histocompatibility antigen-specific T cells and chronic GVHD (Baird, 2010).

Acute GVHD. The three main organs subject to acute GVHD are the skin, the liver, and the gastrointestinal tract. In a comprehensive review, Martin et al. (1990) found that at the onset of acute GVHD, 81 percent of patients had skin involvement, 54 percent had involvement of the gastrointestinal tract, and 50 percent had liver involvement. Experimental data suggest that the lungs might also be targets of acute GVHD (Cooke, 1996). Acute GVHD begins usually 6 or more days posttransplantation (or posttransfusion in the case of nonirradiated blood products) (Anderson & Weinstein, 1990) with high unrelenting fever, a morbilliform maculopapular erythematous rash, and/or severe diarrhea (Glucksberg et al., 1974). The rash becomes progressively confluent and may

involve the entire body surface; it is both pruritic and painful and eventually leads to marked exfoliation. Eosinophilia and lymphocytosis may develop, eventually followed by hepatosplenomegaly, abnormal liver function tests, and elevation in blood conjugated bilirubin levels. The diarrhea is watery and voluminous and may become bloody as the disease progresses. Nausea, vomiting, and severe cramping abdominal pain can occur as a consequence of the gastrointestinal involvement, which is accompanied by a protein-losing enteropathy. The latter contributes to marked generalized edema ("third-spacing") seen in more severe acute GVHD. The most severe forms of acute GVHD are also characterized by bone marrow aplasia, marked susceptibility to infection (Gratama et al., 1987; Parkman, 1991; Skinner et al., 1986), and death in a high proportion of cases.

GVHD is usually mild and self-limited in SCID hosts who receive unfractionated HLA-identical marrow (Parkman, 1991). However, historically, GVHD was found to be moderate to severe despite HLA identity in 60 percent of recipients given pretransplant irradiation or immunosuppressive drugs, and it was fatal in 15 to 20 percent (Deeg & Henslee-Downey, 1990; Glucksberg et al., 1974; Martin et al., 1987). The severity of GVHD increases with the recipient's age (Parkman, 1991; Storb et al., 1986), the use of HLA-matched unrelated donors, and, if a related donor is not HLA-identical, with the degree of genetic disparity.

The staging of acute GVHD is based on the severity and number of organ systems involved. The initial grading criteria were developed by Glucksberg in 1974 (Glucksberg et al., 1974). Most pediatric centers have adopted the modified Glucksberg grading scale (Przepiorka, 1995), which takes into consideration the staging of the GVHD of the gastrointestinal tract based on volume per kg of body weight. The overall grades are classified as I (mild), II (moderate), III (severe), and IV (very severe):

- *Grade I:* 1+ to 2+ skin rash without gut involvement and with no more than 1+ liver involvement
- *Grade II:* 1+ to 3+ skin rash with either 1+ to 2+ gastrointestinal involvement or 1+ to 2+ liver involvement or both
- *Grade III:* 2+ to 4+ skin rash with 2+ to 4+ gastrointestinal involvement with or without 2+ to 4+ liver involvement. Decrease in performance status and fever also characterize grades II and III, with increasing severity per stage.
- *Grade IV:* The pattern and severity of GVHD is similar to those in grade III, with extreme constitutional symptoms.

Chronic GVHD. Historically, GVHD is termed *chronic* when it occurs after 100 days posttransplant. As this arbitrary definition does not consider the clinical manifestations, there is current consensus that clinical features (rather than the timing of their occurrence) should guide the classification in acute, chronic, or "overlap syndrome." Criteria for the diagnosis and staging of chronic GVHD have been proposed by the National Institutes of Health (NIH) Consensus Development Project (Filipovich, 2005).

Chronic GVHD may evolve from acute GVHD (progressive type), develop after resolution of acute GVHD (quiescent or interrupted type), or occur de novo. An overlap syndrome is defined by the concomitant presence of clinical features of acute and chronic GVHD (Choi, 2010). Characterized by a combination of auto- and allo-immune dysregulation with significant immune deficiency, chronic GVHD is an important complication after allogeneic HSCT (Lee, 2003). It is it also the major cause of late mortality in HSCT patients and accounts for significant morbidity (Jacobsohn, 2010).

Although the incidence of chronic GVHD tends to be lower in children (20–50 percent) than in adults (60–70 percent), an increase has been observed in children recently due to the larger use of peripheral blood stem cells and the increased age of transplant recipients (Choi, 2010; Lee, 2003). In children, the rates of chronic GVHD vary from as low as 6 percent in matched sibling cord blood transplants (Rocha, 2000; Wagner, 1995) and 15 percent in recipients of HLA-matched sibling bone marrow transplants (Rocha, 2000) to as high as 65 percent in MUD peripheral blood stem cell transplants (Meisel, 2007). Predisposing factors for the development of chronic GVHD include preceding acute GVHD, older recipient age, male recipient of a female donor graft (in particular if the donor is multiparous), and, as mentioned above, the use of peripheral blood stem cells (Lee, 2003; Zecca, 2002).

Chronic GVHD can affect most organs of the body, most commonly the skin. To confirm the diagnosis of chronic GVHD, at least one distinctive manifestation for chronic GVHD is required, such as oral or vaginal lichenoid findings, ocular sicca, skin dyspigmentation, scleroderma, or bronchiolitis obliterans. Skin lesions of chronic GVHD resemble scleroderma, with hyperkeratosis, reticular hyperpigmentation, atrophy with ulceration, and fibrosis and limitation of joint movement. The gastrointestinal tract, lungs, joints, and liver can be affected as well (Choi, 2010). Chronic GVHD remains a major source of morbidity, decreased quality of life, and mortality in children receiving HSCT. However, it is hoped that the development of new assessment tools and therapies will improve the outcome for children.

Prevention of GVHD. The best approach to GVHD is prevention. Ex vivo T-cell depletion from the donor marrow or blood is the most effective means of limiting both acute and chronic GVHD, but at the cost of serious side effects (such as graft rejection, delayed immune reconstitution, life-threatening infections, and EBV-associated lymphoproliferative disorders). In vivo T-cell depletion uses alemtuzumab, a monoclonal antibody specific for CD52 antigen, which is abundantly expressed on the surface of lymphocytes (Barge, 2006; Kottaridis, 2000), or ATG, a polyclonal antibody (of either horse or rabbit origin) directed against various human T-cell epitopes (Bacigalupo, 2001).

For patients receiving pretransplant chemotherapy prior to unfractionated HLA-identical marrow transplants, GVHD prophylaxis is necessary. The most widely used primary GVHD prophylaxis following full myeloablative conditioning was initially described in 1986 (Storb et al., 1986) and is based on

cyclosporine A associated with a short course of methotrexate (15 mg/M^2 on the first day posttransplantation and 10 mg/M^2 on days 3, 6, and 11). In subsequent years, large randomized studies compared the association of cyclosporine/methotrexate versus tacrolimus/methotrexate and showed no overall survival advantage despite a reduced incidence of grade II to IV acute GVHD with tacrolimus (Horowitz, 1999; Nash, 2000; Ram, 2009). Cyclosporine and tacrolimus are both calcineurin inhibitors impeding the function of the cytoplasmic enzyme calcineurin, which is critical for T-cell activation.

Sirolimus, initially used in solid organ transplantation, has become an attractive alternative prophylactic agent due to its nonoverlapping toxicities with calcineurin inhibitors and the different mechanisms of action (Choi, 2010). GVHD prophylaxis based on the combination of tacrolimus and sirolimus results in rapid engraftment, a reduced incidence of acute GVHD and transplant toxicity, and improved survival (Cutler, 2007).

GVHD prophylaxis in the setting of RIC is based on the association of a calcineurin inhibitor (such as cyclosporine or tacrolimus) with mycophenylate mofetil (MMF) (Mohty, 2004), the prodrug of mycophenolic acid. MMF selectively inhibits the enzyme inosine monophosphatase dehydrogenase, which is needed for the de novo synthesis of guanosine nucleotide and consecutively T-cell proliferation (Choi, 2010). In patients receiving cord blood transplantation, MMF is also often preferred to methotrexate because of the reduced toxicity with regard to neutropenia and mucositis (Choi, 2010).

TREATMENT OF GVHD

Once acute GVHD develops, adequate and timely treatment is necessary irrespective of conditioning. Systemic corticosteroids are widely used as initial treatment, and the primary response to the first-line treatment of acute GVHD remains the most important predictor of long-term survival (Levine, 2010; Weisdorf et al., 1990); efficient treatment is more and more difficult with increasing severity of GVHD. In addition to the above-mentioned drugs, anticytokine and anticytokine receptor antibodies have also been employed to mitigate GVHD (Deeg & Henslee-Downey, 1990); the most useful of these have been antibodies to the IL-2 receptor (anti-CD25) (Srinivasan et al., 2004).

Despite a variety of immunosuppressive and immunomodulatory drugs and second-line treatments, there is no consensus on standard treatment for acute and/or chronic GVHD, and the therapeutic approach is often tailored to the patient and his or her comorbidities. To discuss the numerous treatment options is beyond the scope of this work. Several recent reviews outline the management of GVHD in children (Choi, 2010), with regard to acute (Jacobsohn, 2008) and chronic GVHD (Baird, 2010; Jacobsohn, 2010). Preemptive treatment strategies and innovative approaches aiming at immunomodulation rather than at immunosuppression are needed to ultimately improve long-term outcome in GVHD.

SEQUELAE OF PRETRANSPLANT CHEMOTHERAPY FOR BMT

VOD. VOD is a major cause of mortality following conditioning regimens based on cytoreductive agents, which damage the hepatic vascular endothelium (Eltumi et al., 1993; McDonald et al., 1985). Diagnosis of VOD according to McDonald's criteria requires two of the following: hyperbilirubinemia, hepatomegaly and/or right upper quadrant pain, and weight gain with or without ascites. Distinguishing VOD from other causes of liver damage can be difficult after BMT, particularly when thrombocytopenia poses an unacceptable risk for diagnostic percutaneous liver biopsy in the early posttransplant period. Most often the diagnosis will be made on clinical criteria; however, serum concentrations of the aminopropeptide of type III procollagen (PIIINP) above a standard deviation score of 8 have been predictive of VOD and similarly useful in the diagnosis and monitoring of this condition (Eltumi et al., 1993; Ho, 2008; Pihusch, 2005).

In addition to hepatic damage caused by use of chemotherapeutic agents for pretransplant conditioning, such agents can also result in considerable pulmonary toxicity posttransplantation, leading to prolonged oxygen dependency and to pulmonary arterial hypertension. Often this problem is difficult to distinguish from delayed-onset pulmonary complications involving both the airway and lung parenchyma, which are also observed frequently after HSCT. They include infectious complications in the immunocompromised host (such as CMV pneumonia) but also noninfectious late complications like bronchiolitis obliterans (BO), bronchiolitis obliterans organizing pneumonia (BOOP), and idiopathic pneumonia syndrome (IPS).

Defibrotide, a single-stranded polydeoxyribonucleotide with antithrombotic and fibrinolytic effects on microvascular endothelium, had shown therapeutic efficacy upon early intervention for VOD in a pediatric retrospective multicenter study (Corbacioglu, 2004). Two pediatric nonrandomized studies on the efficacy of defibrotide showed that the VOD incidence and severity were reduced in the defibrotide group compared to a historical control group, suggesting that defibrotide might be effective in preventing and treating VOD (Corbacioglu, 2006; Qureshi, 2008). Randomized trials are now required to definitively establish the role of defibrotide.

Growth retardation and hormonal disturbances. Growth retardation and hormonal disturbances frequently occur in children who are long-term survivors after HSCT for malignancies, who were given specific chemotherapy prior to pretransplant conditioning (Clement-De Boers et al., 1996; Ranke, 2005). In this setting, sterility is common (Sanders, 1996). There are fewer long-term data available for patients who received HSCT for inborn errors of the immune system. In 149 SCID patients treated by allogeneic HSCT between 1972 and 2004 at Necker Hospital, Paris, France (Neven, 2009), showed that 15 percent of the 94 long-term survivors presented with growth failure, with a weight and height below 2 standard deviations at some point in their follow-up longer than 2 years after HSCT. Concomitantly, significant clinical events (such as autoimmune and inflammatory complications

or chronic GVHD) were noted in all but one of these patients, and growth failure improved with resolution of the concomitant clinical events in all but one. No growth-hormone deficiency was found in the analyzed patients, whereas thyroid-hormone deficiency was found in 2 patients. In only 3 of the 39 patients aged older than 15, adult weight or height were below –2 standard deviations. All patients aged 15 and older have completed clinically normal pubertal development (Neven, 2009).

IMMUNE RECONSTITUTION AFTER BMT

BMT for SCID

BMT has been more widely applied and was more successful in infants with SCID than in any other primary immunodeficiency (Antoine et al., 2003; Buckley, 2004, 2011; Buckley et al., 1999; Friedrich, 1996; Gennery et al., 2010; Haddad et al., 1998). Since the first such transplant was done, more than 1,200 patients with SCID worldwide have received transplants of HLA-identical or haploidentical bone marrow or of unrelated marrow or cord stem cells (Antoine et al., 2003; Buckley, 2011; Cowan, 2008; Gennery et al., 2010; Grunebaum, 2010; Neven, 2009).

A recent survey of the European Group for Blood and Marrow Transplantation and the European Society for Immunodeficiency reported on 699 infants with SCID who had received BMT from 1968 to 2005 (Table 60.2). The proportions of the different genetic forms of SCID remained stable over time. Two hundred three patients had received transplants from a genotypically (135) or phenotypically (68) HLA-identical relative and 81 from a MUD, whereas 415 had received T-cell–depleted haploidentical related transplants (Gennery et al., 2010; Fig. 60.1).

The preceding European report on 566 transplantations in 475 SCID patients during the period from 1968 to 1999 showed that the 3-year survival with sustained engraftment was significantly better after HLA-identical than after mismatched transplantation (77 percent vs. 54 percent ; $p = 0.002$) (Antoine et al., 2003). These findings were in line with previously published survival rates for SCID patients receiving transplants at the two largest centers in Europe, Hopital Necker in Paris (Stephan et al., 1993) and the University of Ulm (Friedrich et al., 1993): 80 percent for HLA-identical unfractionated marrow recipients and approximately 55 percent for recipients of haploidentical, T-cell–depleted marrow. Similar survival statistics had also been reported by SCID transplant centers in the United States (Dror et al., 1993; O'Reilly, 1991; O'Reilly et al., 1986).

The most recent European data show that the survival continues to improve over time, reaching a survival rate of about 90 percent for transplantations for patients with SCID when using a geno-identical sibling donor (Gennery et al., 2010). The outcome for selected patients (newborns without any preexisting infection) is even better (Brown, 2011; Myers et al., 2002), underlining the fact that neonatal screening for SCID may significantly improve the outcome for SCID patients (Buckley, 2012). One of the most significant findings

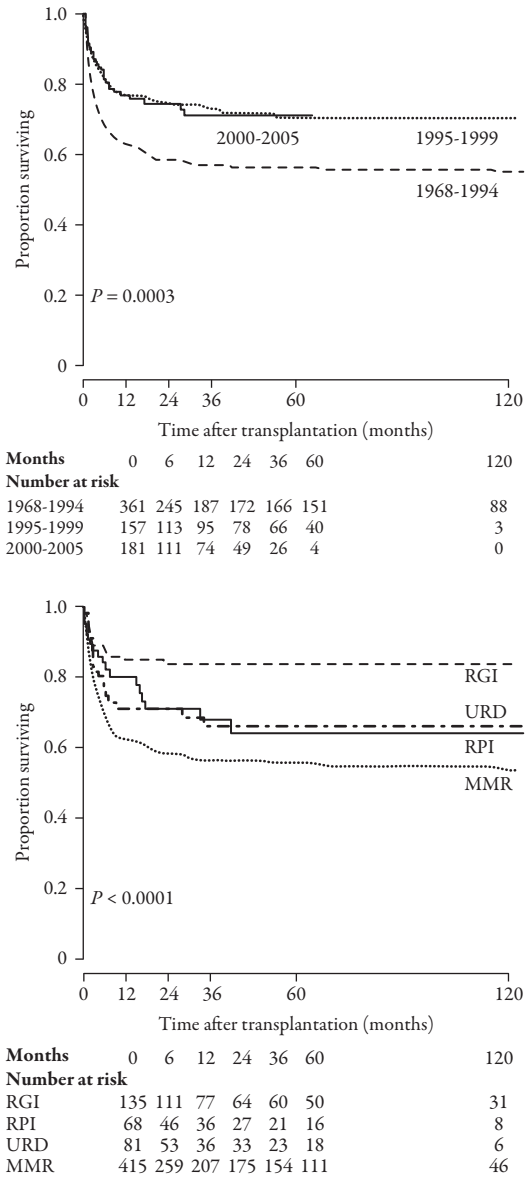


Figure 60.1 Cumulative probability of survival in patients with SCID after HSCT according to to the period in which transplanted, donor source (related or URD), HLA matching. MMR, Mismatched related; RGI, related genoidentical; RPI, related phenoidentical and whether B cells were present or absent in the SCID patients. (From Gennery et al, *J Allergy Clin Immunol* 126(3):602-10, 2010, with permission)

from this European investigation was that there is no longer a significant advantage for a MUD over an HLA-mismatched related donor for SCID patients transplanted between 2000 and 2005, with 3-year survival rates of almost 70 percent in both cases (69 percent for MUD vs. 66 percent in mismatched related donor). This is in contrast to previous reports that showed a clear survival advantage for MUD over haploidentical donors in SCID recipients (survival rate of 80.5 percent vs. 52.5 percent respectively) (Grunebaum, 2006), and it is due to the improved survival of the patients with haploidentical donors, thus enabling clinicians to proceed rapidly to transplantation even if there is no geno- or pheno-identical familial donor. The number of MUD transplants increased over time:

while only 28 patients with SCID were reported in the former European series as having received MUD transplants between 1968 and 1999, and 63 percent were surviving (Antoine et al., 2003), there were 46 MUD transplants in the period from 1999 to 2005, and 69 percent were surviving (Gennery et al., 2010). The steady improvement over time in the survival rates of SCID transplant recipients is most likely due to early diagnosis (Figure 60.3) and more effective prevention or treatment of disease-related and transplantation procedure complications, notably infections and GVHD. The improvement over time of the HSCT outcome in SCID patients is a major accomplishment, since SCID is 100 percent fatal without transplantation or enzyme replacement therapy, helpful only for ADA deficiency, approximately 16 percent of SCID (Buckley, 2011; Buckley et al., 1997; Stephan et al., 1993).

RHB recently reported the long-term outcome, according to molecular type, of 166 consecutive SCID infants who had received nonconditioned related donor bone marrow transplants at Duke University Medical Center over 28.3 years, the largest single center experience in the world (Buckley, 2011). Age at diagnosis ranged from newborn to 21 months. The grafts were T-cell–depleted in the 149 HLA-haploidentical and also in 9 of the 17 HLA-identical transplants. Forty-nine patients received from one to three additional T-cell–depleted marrow transplants, from either the original donor or another haploidentical relative. No pretransplant conditioning or posttransplant GVHD prophylaxis was given, except in two patients who received cyclosporine for 1 month because of cutaneous GVHD from transplacentally transferred maternal T cells at presentation. Five of the infants who received haploidentical marrow transplants were also given unrelated placental blood transplants. Four of the latter received pretransplant conditioning, and they were also given posttransplant prophylaxis against GVHD. The overall survival rate was 76 percent (Figure 60.2). Of the 126 survivors, 125 had survived 1 or more years after transplantation, 110 were alive 5 or more years, and 83 had survived for 10 or more years. Median follow-up of surviving patients was 10 years.

Survival of the 48 infants transplanted during the first 3.5 months of life was significantly higher (94 percent) than the survival of the patients who were transplanted after that age (69 percent; $p = 0.001$). Thirty of the 40 deaths were due to viral infections. GVHD, usually Grade 1 and not requiring treatment, occurred in 45 of the 149 patients given T-cell–depleted haploidentical parental marrow, in 8 of 17 given HLA-identical marrow, and in 4 of 5 given placental blood (Buckley, 2011).

The long-term outcome of immune reconstitution in these patients was reported earlier showing that T cell function, T cell diversity and thymic output continued into the third decade despite the lack of pre-transplant conditioning, supporting the possibility of stem cell engraftment (Sarzotti-Kelsoe et al., 2009). In addition, to determine the long-term clinical outcome, a questionnaire was sent to the parents of the survivors and the patients were also seen in clinical follow-up as well. There were few patients with serious health problems, none with chronic or severe GVHD, few with autoimmune problems, and 86% were considered healthy by their families (Railey et al., 2009). Many had gone to college or graduate school, including one who was in medical school. Four of the survivors had become parents of normal children.

Problems encountered with T-cell–depleted haploidentical transplants. The period of vulnerability to infection is possibly longer in recipients of T-cell–depleted marrow compared to those who receive unfractionated HLA-identical marrow because of the 90 to 120 days required for donor stem cells to develop into T cells in the recipient's thymus (Buckley et al., 1986) and the fact that there are no adoptively transferred donor T cells. However, because patients who receive unfractionated transplants usually receive GVHD immunosuppressive agents for many months posttransplantation, they are also at risk for infection. Prior to the introduction of anti-CD20 antibodies (Rituximab), the occurrence of B-cell lymphoproliferative disease was a major problem with this type of transplantation, whereas it did not usually occur in recipients of unfractionated HLA-identical marrow (Filipovich et al., 1994; Levine, 1994; Shearer et al., 1985; Trigg et al., 1985).

Posttransplant lymphoproliferative disorder (PTLD) caused by EBV has often been fatal in high-risk allogeneic HSCT recipients. The development of monoclonal antibodies and EBV-specific cytotoxic T lymphocytes (CTLs) has considerably improved the treatment options for EBV-PTLD, allowing a balance between outgrowing EBV-infected B cells and the EBV CTL response, or targeting the B cells with monoclonal antibodies or chemotherapy (Heslop, 2009). It has been shown that preemptive therapy with rituximab in children is safe but only partly effective in haploidentical HSCT recipients (Comoli, 2007). Infusion of EBV-specific CTLs can further rescue patients who progress to PTLD under rituximab treatment. A recent review showed that preemptive use of rituximab and EBV-specific CTLs significantly reduced the risk of death due to EBV-PTLD in HSCT recipients, with survival rates of 89.7 percent and 94.1 percent, respectively (Styczynski, 2009).

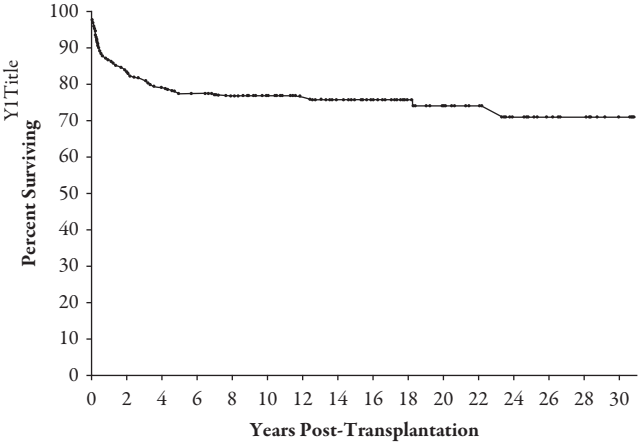


Figure 60.2 Thirty-one year survival (76%) of 173 SCID patients given non-ablated, related donor bone marrow transplants at Duke University Medical Center: 18 HLA-identical, 155 HLA Haploidentical.

Other problems encountered following haploidentical transplants include resistance to engraftment and frequent failure to obtain engraftment of donor B cells or to develop B-cell function. The first author (R.H.B.) has attempted to overcome both of the latter two problems by performing nonablative "booster" transplants. A study was conducted of 49 SCID patients (28.7 percent of 171 SCIDs transplanted over 30 years) who had received booster transplants to define the long-term outcome, factors contributing to a need for a booster and factors that predicted success (Teigland et al., 2013). Of the 49 patients, 31 (63 percent) are alive for up to 28 years. Age at initial transplantation was found to have a significant effect on outcome (mean of 194 days old for living patients, versus a mean of 273 days old for deceased patients, $p = 0.0401$). Persistent viral infection was present in most deceased booster patients. In several patients, the use of two parents as sequential donors resulted in striking T and B cell immune reconstitution. A majority of the living patients have normal or adequate T-cell function and are healthy. The development of adoptive immunotherapies or novel approaches to accelerate de novo thymopoiesis is currently receiving much attention (Reimann, 2010). Strategies to hasten posttransplant immune reconstitution without triggering GVHD such as the recently reported adoptive transfer of regulatory T cells in haploidentical recipients, which was shown to be able to prevent GVHD, promote lymphoid reconstitution, and improve immunity to opportunistic infections (Di Ianni, 2011).

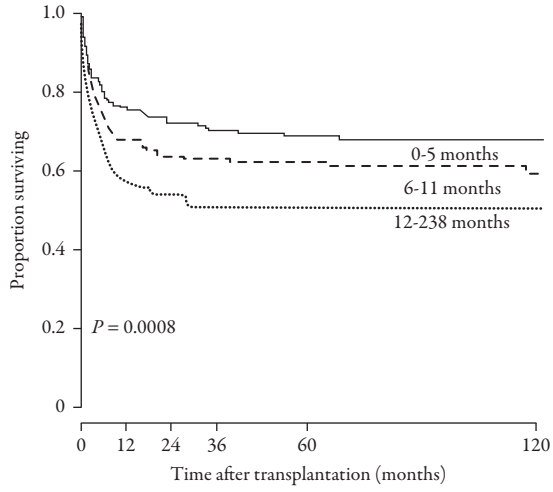
Kinetics of development of immune function. The time to development of immune function following haploidentical stem cell grafts is quite different from that after unfractionated HLA-identical marrow. In contrast to the rapid emergence of immune function within 2 weeks after the latter type of grafts due to adoptive transfer of donor T, B and NK cells, lymphocytes with mature T-cell phenotypes and functions fail to appear in significant number until at least 3 to 4 months after transplantation of T–cell-depleted marrow (Buckley et al., 1986; Dror et al., 1993; Moen et al., 1987; Wijnaendts et al., 1989). This same result holds true even if pretransplant conditioning is given (Friedrich et al., 1993) or if T-cell–depleted, HLA-identical sibling marrow is used (R.H.B., unpublished). Thus, regardless of whether the donor is HLA-identical or -haploidentical, it takes between 90 and 120 days for stem cells in T-cell–depleted marrow to mature into functioning T cells in SCID infants (Buckley et al., 1986). The reason for this delay is not known, but presumably this is how long thymic maturation of stem cells takes. This period of time is not unlike that in intrauterine life, in which PHA-responsive cells do not appear until around 12 weeks of gestation. The first function to develop after transplantation is NK-cell function. Shortly after NK cells, T-cell function appears, simultaneously with the development of phenotypically normal T cells. Chimerism studies reveal that such T cells are always 100 percent of donor origin (Buckley et al., 1999). Thus, the tiny architecturally abnormal epithelial thymi of all forms of SCID treated thus far appear to have the ability to cause normal stem cells to become phenotypically and functionally normal T cells (Patel et al., 2000; Myers et al., 2002; Sarzotti-Kelsoe et al., 2009). These observations also indicate that the thymic microenvironment of most infants with SCID is capable of differentiating not only fully matched but also half-matched normal stem cells to become mature and functioning T lymphocytes and long-term follow-up studies have shown that this continues even into the third decade post-transplantation (Sarzotti-Kelsoe et al, 2009).

Damage to the thymic epithelium due to pre-HSCT conditioning, viral infections and acute and/or chronic GVHD may cause delayed and incomplete T-cell reconstitution (Cavazzana-Calvo, 2009; Olkinuora, 2011). Genetic analyses of the cells from such chimeric patients have revealed that all T cells are of donor origin, whereas the B cells, antigen-presenting cells, neutrophils, platelets, and red cells remain those of the recipient in a large majority of cases unless pretransplant conditioning is given (Brady et al., 1996; Buckley et al., 1986; Friedrich et al., 1993; Schouten et al., 1988; van Leeuwen et al., 1994; Vossen et al., 1993).

B-cell function develops much less often and some patients fail to develop B-cell function altogether, despite normal T-cell function (Buckley, 2004; Buckley, 2011 ; Buckley et al., 1986, 1999; Dror et al., 1993; Giri et al., 1994; Moen et al., 1987; Neven, 2008; Wijnaendts et al., 1989).

An extensive review of B-cell reconstitution (as presented in 19 reports from the United States and Europe on posttransplantation immune reconstitution in patients with SCID over the most recent two decades) was performed by R.H.B. (Buckley, 2011). The finding was that while the use of pretransplantation chemoablation may result in a higher percentage of B-cell chimerism and function it did not guarantee normal B-cell function, as a significant percentage of patients required long-term immunoglobulin replacement. Moreover, the mortality was much greater at those centers that used pretransplant conditioning.

The first author and her colleagues have recently reported the results of analyses of B cell function in 125 surviving SCID marrow recipients at Duke University Medical Center prior to and long-term after non-ablative BMT, according to their molecular type (Buckley et al., 2013). Studies included blood immunoglobulin measurements; antibody titers to standard vaccines, blood group antigens and bacteriophage Φ X 174; flow cytometry to examine for markers of B cell immaturity, memory, switched memory B cells and BAFF receptor expression; B cell chimerism; B cell spectratyping; and B cell proliferation. The results showed that B cell chimerism was not required for normal B cell function in IL7Rα-Def, ADA-Def and CD3-Def SCIDs. In X-linked-SCID, Jak3-Def SCID and those with V-D-J recombination defects, donor B cell chimerism was necessary for B cell function to develop. Thus, the most important factor determining whether B cell function develops in SCID T cell chimeras is the underlying molecular defect. In some types, host B cells function normally once T cells develop. In those molecular types where host B cell function did not develop, donor B cell chimerism was necessary to achieve B cell function.



Months	0	6	12	24	36	60	120
Number at risk							
0-5 months	289	210	158	135	118	92	47
6-11 months	253	170	134	110	94	70	32
12-238 months	145	80	56	48	40	28	12

Figure 60.3 Cumulative probability of survival in patients with SCID after HSCT according to age at transplantation through all periods. (From Gennery et al., *J Allergy Clin Immunol* 126(3):602–10, 2010, with permission)

BMT FOR IMMUNODEFICIENCIES OTHER THAN SCID

Allogeneic BMT has been found to be effective in conferring improved immune function in an increasing number of genetically distinct immunodeficiencies other than SCID (Tables 60.3 and 60.4) (Buckley & Fischer, 1999; Fischer et al., 1986, 1990, 1994; Friedrich, 1996; Le Deist et al., 1989; Szabolcs, 2010). A recent survey from the European Group for Blood and Marrow Transplantation and the European Society for Immunodeficiency analyzed the outcome of 783 patients with various forms of non-SCID primary immunodeficiencies who had received transplants from 1968 to 2005 (Gennery et al., 2010).

Indications. Indications for BMT in non-SCID primary immunodeficiency obviously depend on a critical assessment of life expectancy, as well as quality of life in a given setting. Disease prognosis alone is not usually sufficient to lead to such a decision. Age, past clinical history, and sequelae of illnesses (chronic viral infection, immunosuppressive treatment required for control of autoimmunity, organ dysfunction), clinical course of previously affected family members, gene mutation determination when available, context of medical care (distance from specialized centers), and psychological assessment of the child and the family are some of the many factors to be taken into consideration when attempting to decide for or against BMT.

As greater experience with BMT has been gained, many of the factors leading to increased mortality and morbidity have decreased with time. It is not appropriate to assess the risks associated with BMT based on results reported in the early 1980s. For instance, in Europe, the rate of success in BMTs performed since October 1985 (up to March 1991) was 81.5, versus 52 percent before October 1985 (Antoine et al., 2003). A specific example is seen in the case of chronic granulomatous diseases, where recent comparisons of conservative treatment versus BMT have led to a change in attitude (see below). Over the past few years, the use of curative HSCT in early childhood has expanded due to the impressive results achieved for matched, unrelated, or cord blood donors after RIC (Seger, 2010). These remarks emphasize the need for careful updating of BMT registries to aid the medical community in making decisions about patients with primary immunodeficiency diseases.

BMT IN NON-SCID IMMUNODEFICIENCIES

BMT has not been as uniformly effective in the treatment of patients with non-SCID T-cell immunodeficiencies as it has in SCID (Fig. 60.4). Such patients have somewhat less severe immune dysfunction than do those with SCID, and this enables them to reject marrow grafts. However, an increasing number of transplants with HLA-identical sibling marrow or matched unrelated adult marrow, peripheral blood stem cells, and cord blood have been successful after chemoablation (Tables 60.3 and 60.4).

The recent survey from the European Group for Blood and Marrow Transplantation and the European Society for Immunodeficiency on 783 non-SCID patients shows an improved 3-year survival for genotypically and phenotypically HLA-identical related donors: 79 percent and 76 percent respectively for the period from 2000 to 2005 compared to 71 percent and 53 percent for the period from 1995 to 1999 and 72 percent and 46 percent for the period before 1995 (Gennery et al., 2010).

Following the improved results with T-cell–depleted HLA-haploidentical BMT for the treatment of SCID, this approach was also tried for the treatment of other lethal immunodeficiency diseases. In contrast to the significant improvement in the survival rate of SCID patients receiving a haploidentical T-cell–depleted graft, which now reaches 66 percent, the outcome of haploidentical HSCT in non-SCID patients is still poor: the 3-year survival was 55 percent in the last European survey for the period from 2000 to 2005 (compared to 41 percent for the period before 2000) (Gennery et al., 2010).

Delay in the development of T- and B-cell function (6–40 months) causes significant infectious complications that remain a major concern in recipient of T-cell–depleted HLA-haploidentical transplants. As discussed above, the development of new immunotherapies may contribute to improve the outcome of high-risk haploidentical transplants in non-SCID patients. Because of the poor outcome of haploidentical grafts, a search for a MUD should be undertaken in the absence of HLA-matched siblings.

Wiskott-Aldrich syndrome (see Chapter 43). This defect is the second most common primary immunodeficiency for which BMT has been applied (for a recent review see Pai,

Table 60.3 IMMUNODEFICIENCIES OF THE LYMPHOID SYSTEM THAT ARE CURABLE BY ALLOGENIC HSCT

Adenosine deaminase deficiency
Purine nucleoside phosphorylase deficiency
X-linked SCID (γ c deficiency)
AR* T-B+ SCID (JAK3, IL-7Rα, CD45, CD3δ/CD3ε,/CD3ζ, Coronin-1A)
AR* T-B- SCID (Rag1/Rag2, Artemis, DNA-Pkcs)
Omenn Syndrome (hypomorphic mutations in Rag1/Rag2, Artemis, IL-7Rα, RMRP,
ADA,DNA-Ligase IV, γc)
DNA ligase IV deficiency
Cernunnos deficiency
Reticular Dysgenesis
Defective T cell activation (including CD8 deficiency, ZAP70 deficiency, Calcium flux
deficiency, and cytokine production deficiency)
MHC class II deficiency
Cartilage hair hypoplasia
Wiskott-Aldrich syndrome
CD40/CD40 ligand deficiency
X-linked lymphoproliferative syndrome (XLP)
X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) and type 2
(XLP-2/XIAP deficiency)
Autoimmune lymphoproliferative syndrome with Fas (CD95) deficiency
ITK deficiency
DOCK8 deficiency

*AR, Autosomal recessive.

2010). After only partial correction was achieved with the first BMT given to a WAS patient in 1968 (Bach et al., 1968), full correction was achieved in other WAS patients in 1978 by use of a more effective conditioning regimen (Parkman et al., 1978). Transplantation has since been successful in 85 to 90 percent of patients who had an HLA-identical sibling donor (Tables 60.2 and 60.5) (Brochstein et al., 1991; Ozsahin et al., 1996). In the absence of an HLA-identical donor, T-cell–depleted haploidentical marrow transplants have been tested: Rumelhart et al. (1990) reported success in three of four WAS patients, whereas in another series only one of six survived (Brochstein et al., 1991). MUD bone marrow, peripheral blood, and cord blood transplants have been also successful (Knutsen et al., 2003; Lenarsky et al., 1993; Miano et al., 1998; Pai, 2006).

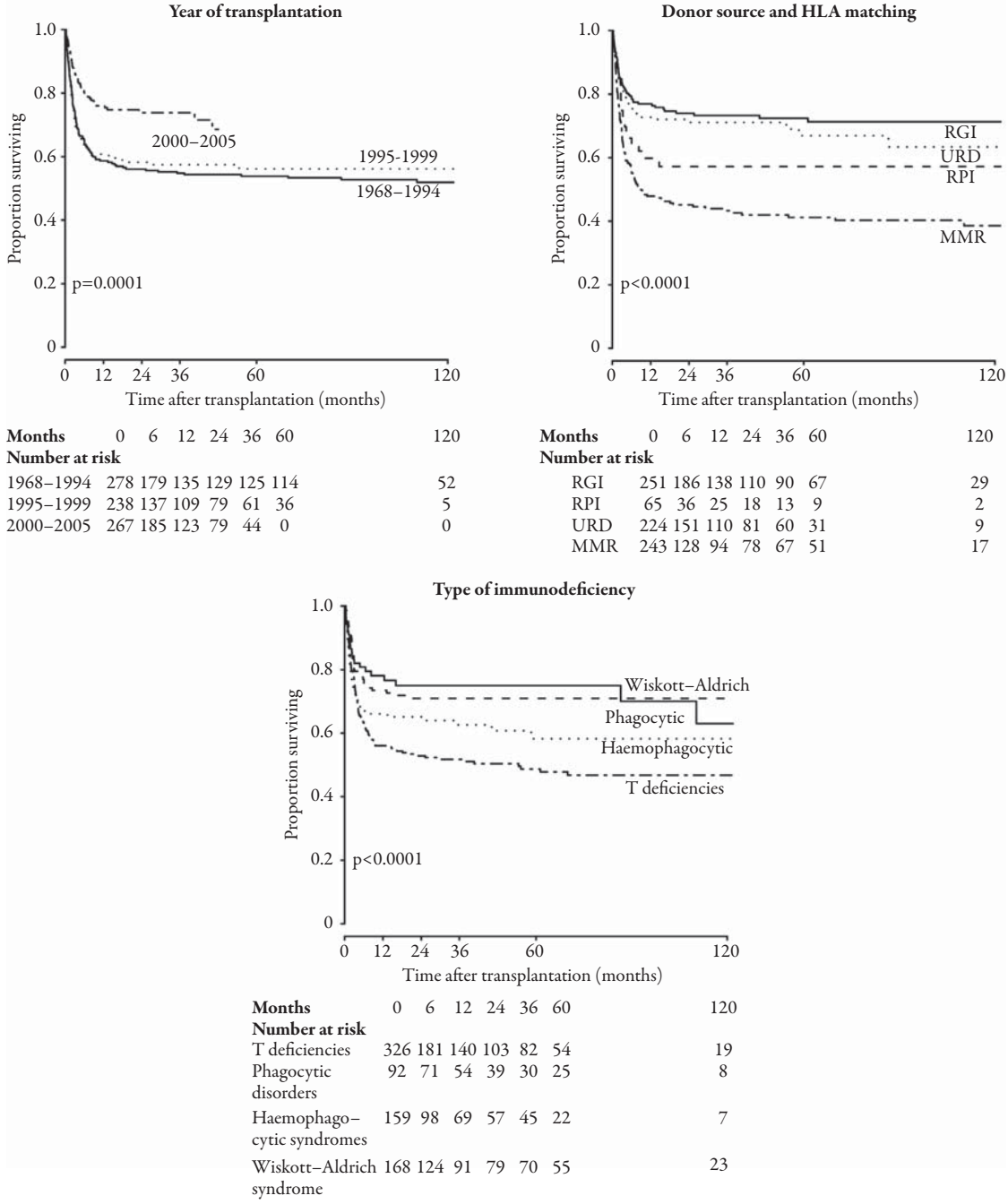
Table 60.4 IMMUNODEFICIENCES OF THE PHAGOCYTIC SYSTEM CURABLE BY ALLOGENEIC BONE MARROW TRANSPLANTATION

Chronic granulomatous diseases (X-linked and AR)
Leukocyte adhesion deficiency type I
Chediak-Higashi syndrome
Immunodeficiency with partial albinism (Griscelli disease)
Familial hemophagocytic lymphohistiocytosis
Interferon γ receptor deficiencies
Severe congenital neutropenia and other neutropenias (cyclic neutropenia, Shwachmann syndrome)

Recently, Friedrich et al. reported on 39 WAS patients treated by HSCT since 1983 (15 transplants from HLA-identical unrelated donors, 15 from mismatched parental donors, and 9 from matched siblings). The overall survival rate was 90 percent in patients with matched donors and 50 percent in patients after nonidentical transplantation, with a mean follow-up time of 11 years. Treatment failures in the latter group were mainly related to graft rejections and to GVHD and infections following repeat transplants (Friedrich, 2009). Other series have reported an overall survival of 78.2 percent (18/23) in a cohort of 23 WAS patients (including 16 MUD) and a survival of 81.2 percent (13/16) of MUD BMT recipients (Pai, 2006). Other single-center reports show a survival of 69 percent in 13 patients (Munoz, 2007) and a 5-year failure-free survival rate of 65.7 percent in 57 patients (Kobayashi, 2006).

There are also a few reports of the use of unrelated umbilical cord blood stem cell transplantation in patients with WAS (n = 3, Knutsen, 2003; n = 1, Diaz de Heredia, 2008; n = 15, Kobayashi, 2006), showing that unrelated umbilical donor cord blood may be a valid option for children with WAS lacking an HLA-identical sibling donor.

Due to the rarity of this disorder, multicenter studies are mandatory to accurately evaluate the outcome with regard to the different donor sources. In a report from the International Bone Marrow Transplant Registry, 170 WAS patients had been transplanted, and the 5-year probability of survival (95 percent confidence interval) for all WAS subjects was 70 percent (63–77 percent) (Filipovich et al., 2001). Probabilities differed by donor type: 87 percent (74–93 percent) with HLA-identical sibling donors, 52 percent (37–65 percent) with other related donors, and 71 percent (58–80 percent) with MUD (p = 0.0006). Boys who had received a MUD transplant before age 5 had survivals similar to those receiving HLA-identical sibling transplants. The European experience from a similar timeframe demonstrated 81 percent survival of 32 matched sibling donor recipients and only 45 percent survival of 43 mismatched related transplant recipients during the period from1968 to 1999 (Antoine, 2003). In the following years, from 2000 to 2005, only 3 mismatched related transplants were carried out in a total of 49 HSCTs for WAS, while there was a significant increase in MUD transplants (n = 35) (Gennery et al., 2010). Due to accumulating experience with MUD transplants and advances in donor–recipient matching allowing improved selection of unrelated donors, 77.7 percent of larger centers routinely offered unrelated donor transplants for WAS patients, as revealed by an international survey of 73 centers in 2002 (Conley, 2003).



Months	0	6	12	24	36	60	120
Number at risk							
1968–1994	278	179	135	129	125	114	52
1995–1999	238	137	109	79	61	36	5
2000–2005	267	185	123	79	44	0	0

Months	0	6	12	24	36	60	120
Number at risk							
RGI	251	186	138	110	90	67	29
RPI	65	36	25	18	13	9	2
URD	224	151	110	81	60	31	9
MMR	243	128	94	78	67	51	17

Months	0	6	12	24	36	60	120
Number at risk							
T deficiencies	326	181	140	103	82	54	19
Phagocytic disorders	92	71	54	39	30	25	8
Haemophago–cytic syndromes	159	98	69	57	45	22	7
Wiskott–Aldrich syndrome	168	124	91	79	70	55	23

Figure 60.4 Cumulative probability of survival in non-SCID patients, according to donor source (related or unrelated donor) and HLA matching and type of immunodeficiency through all periods. MMR, Mismatched related; RGI, related genoidentical; RPI, related phenoidentical. (From Gennery et al., *J Allergy Clin Immunol* 126(3):602–10, 2010, with permission).

All aspects of WAS are corrected by marrow transplantation, including the eczema, autoimmunity, and risk of lymphomas. This indicates that these disease complications were related to the immunodeficiency. Similarly, thrombocytopenia and the bleeding tendency disappeared after successful BMT. With increasing numbers of successfully transplanted patients, the long-term outcome, including quality of life, is receiving more and more interest. A recent European long-term, retrospective, multicenter study reported on 96 WAS patients who received transplants between 1979 and 2001 and survived at least 2 years following HSCT (Ozsahin, 2008). The most striking finding was the observation of chronic GVHD-independent autoimmunity in 20 percent of patients. This fact was strongly associated with a mixed/split chimerism status ($p < 0.001$), suggesting that residual host lymphocytes mediate autoimmune disease despite the presence of donor lymphocytes. Furthermore, patients younger than 2 years of age at HSCT (including also those with a mismatched related donor) had a higher chance of long-term survival and a better quality of life than older patients (Ozsahin, 2008).

Important progress has been achieved with regard to survival. Bone marrow, peripheral blood stem cell, or cord blood transplantation for patients with WAS should be performed as early in life as possible, especially in the presence of an HLA-identical sibling. Matched unrelated marrow, peripheral blood stem cells, or cord blood transplants can be effective if performed before the age of 5 years. The poor results with T-cell–depleted HLA-haploidentical BMTs in this condition stress the need for careful evaluation of whether to even attempt HLA-haploidentical BMT in WAS patients who lack both HLA-identical related and unrelated donors (Fischer et al., 1994). Nevertheless, WAS patients who develop refractory thrombocytopenia and/or severe autoimmune manifestations should be considered for HLA-haploidentical BMT.

HSCT FOR OTHER T-CELL DEFICIENCIES, PHAGOCYTIC CELL DISORDERS, AND HEMOPHAGOCYTIC SYNDROMES

An increasing number of patients with other inborn errors of the immune system have become eligible for HSCT, as discussed in detail in the specific chapters on the different genetic conditions. Due to important progress with regard to HSCT procedures and supportive care, there will be more and more patients in whom transplantation is no longer contraindicated. As with all types of transplants in infants with SCID (Fig. 60.1 and 60.2), the long-term (10-year) outcome of HLA-identical BMT for most forms of primary immunodeficiency appears reasonably satisfactory. Correction of the underlying deficiency is sustained despite frequent mixed chimerism in the absence of TBI usage. Sequelae are generally limited to consequences of the primary disease.

Continued international studies are needed to carefully assess the best therapeutic strategies for a given disease without neglecting the long-term outcome, including quality of life.

FUTURE OF HSCT IN THE TREATMENT OF PRIMARY IMMUNODEFICIENCY

It is abundantly clear that allogeneic HSCT cures many children with life-threatening immunodeficiency diseases. Until gene therapy is perfected, it will remain the standard of care for such conditions. Thus far, HSCT seems to be associated with acceptable long-term sequelae, although outcome beyond a follow-up of 30 to 40 years is not yet known. However, some of the long-term survivors have had chronic GVHD and extensive lesions of verruca vulgaris (Laffort et al., 2004; Neven, 2009). Except in the case of SCID, major problems still exist for the many patients who lack an HLA-identical sibling. Stem cell and lymphocyte cell manipulation could provide a better chance of success in the future, while alternative therapies might be developed for more diseases. Better assessment of genetic mutations associated with diseases, as well as individual cofactors, should also lead to a better delineation of which child should receive a transplant.

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61.

GENE THERAPY

Fabio Candotti and Alain Fischer

INTRODUCTION

As currently practiced, gene therapy can be defined as "the transfer of exogenous genes to somatic cells of a patient in order to correct an inherited or acquired gene defect, or to introduce a new function or trait." In the past two decades, gene therapy has moved from the speculative stage to clinical applications as clinical gene transfer protocols have been applied to inherited genetic defects, malignancies, and infectious diseases. Primary immunodeficiency diseases (PIDs), especially severe combined immunodeficiencies (SCID), have played a major role in this process as they have provided a proving ground for the first therapeutic applications (Blaese et al., 1995) as well as the first evidence that gene therapy can be curative (Aiuti et al, 2002a; Cavazzana-Calvo et al, 2000). Several advantages have made PIDs attractive candidate diseases for gene therapy. First, they are often curable by allogeneic hematopoietic stem cell transplantation (HSCT), which translates into the possibility that they should be also treatable with procedures combining ex vivo manipulation and reinfusion of autologous, gene-corrected hematopoietic stem cells. Second, the target cells reside in the bone marrow, a readily accessible tissue, which makes them amenable to ex vivo gene transfer, the procedure that currently offers the best chance of success. In addition, the regulation of genes responsible for many PIDs (i.e., adenosine deaminase [ADA] deficiency, chronic granulomatous disease [CGD], X-linked severe combined immunodeficiency [X-SCID], and leukocyte adhesion deficiency [LAD]) is relatively simple, and while currently available technology does not allow easy reproduction of the physiological control mechanisms of gene expression, the available gene transfer vectors are more than adequate to provide expression of these genes at levels required for clinical benefit. Finally, for several PIDs, gene-corrected progenitors and mature cells will have a survival advantage over the unmodified, affected cell populations. This selective survival translates into the very advantageous possibility that even low gene transfer efficiency may have therapeutic effects.

Because of the extensive recent progress in their molecular and biological characterization, several PIDs also fulfill the general prerequisites for any gene therapy approach that are based on the comprehensive knowledge of the molecular and cellular basis of the candidate disease. In general, the sequence of the cDNA of the gene responsible for the disease represents one critical piece of information for preliminary experiments of corrective gene transfer; however, the elucidation of the genomic organization and the characteristics of the promoting element(s) are also of great practical importance. Moreover, the specific function(s) and regulatory mechanisms of expression of the gene of interest should be clearly understood and the potential deleterious effects of overexpression investigated. Where possible, the safety and efficacy of gene transfer protocols should be tested on animal models, either naturally occurring or experimentally generated. Finally, it is fundamental to verify that the correction of the genetic defect can induce the restoration of gene function(s) in affected cells.

The complete and careful consideration of these issues is critical for development of new gene therapy strategies and should help defining the answer for the ethical dilemma that is proposed whenever the "traditional" treatment for the candidate disease is already highly successful.

Performing gene therapy for PIDs involves a series of specific difficulties. As mentioned, the ultimate target of corrective gene transfer for PIs is the hematopoietic stem cell (HSC). Targeting the HSC is an exceptionally challenging task for several reasons. First, cells demonstrating the characteristics of "true" hematopoietic progenitors are extremely infrequent in the hematopoietic tissues and have thus far eluded

all attempts to characterize their specific cellular phenotype, making their collection and enrichment laborious and inefficient. In addition, HSCs are mostly quiescent and therefore resistant to the insertion of genetic material mediated by viral vectors requiring active cell division. Extensive studies have been dedicated to the identification of "ex vivo" culture conditions that could stimulate HSCs to proliferate, thus making them susceptible to gene integration, without inducing differentiation and loss of long-term repopulating ability. Cytokines and growth factors like interleukin (IL)-3, IL-6, stem cell factor (SCF), thrombopoietin, and Flt-3 ligand, together with culture supports such as fibronectin, have been demonstrated to be of primary importance to this goal (Dao et al., 1997; Hanenberg et al., 1996; Kohn, 1999; Piacibello et al., 1997, 1999; Zandstra et al. 1997). Culture conditions using these reagents have resulted in major improvements in our ability to efficiently introduce genes into HSCs in large animal models (Kiem et al., 1998; Tisdale et al., 1998). However, the current levels of gene transfer may still not be adequate for achieving therapeutic effects in hematopoietic diseases in which corrected cells will not have a strong selective advantage over the mutated ones (e.g., thalassemias or sickle cell disease). On the other hand, this progress has resulted in successful clinical protocols for X-SCID, ADA deficiency, and Wiskott-Aldrich syndrome (WAS) (Aiuti et al., 2002a; Boztug et al., 2010; Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002), thus forming the basis for future applications to other PIDs.

Another obstacle to gene therapy for PIDs is that several of the causative genes that have been identified have expression restricted to specific hematopoietic elements or particular cellular phases of cell development (Table 61.1). In most gene transfer vectors currently available for clinical use, the gene of interest is under the transcriptional control of strong viral promoters and is therefore expected to give rise to unregulated expression of the such gene in transduced HSCs and all deriving cellular elements. This strong, ubiquitous promoter activity raises the concern that constitutive expression of an ectopic gene could have detrimental consequences on the differentiation and/or function of targeted populations. The need for precise regulation of gene expression represents a formidable challenge for the development of gene therapies. The use of gene-specific promoting and/or enhancing elements can be exploited to obtain regulated and/or tissue-specific expression of the therapeutic sequences, as shown for the WAS cDNA (Martin et al., 2005). Alternatively, inducible or repressible systems could also be utilized to modulate gene expression.

Finally, patients with inherited disorders of immunity may present with complete lack of expression of the affected gene(s) or show detectable levels of nonfunctional (mutated) gene product(s). It is not obvious which one of these two possibilities represents the better scenario for successful gene therapy. In the case of subjects carrying "null" mutations, there is the possibility that the newly expressed molecule could induce an immune response resulting in the elimination of the transduced, therapeutic cell populations; on the other hand, in the presence of mutations allowing for the expression of an inactive gene products, the mutated molecules could manifest dominant-negative effects in the presence of the correct protein(s), generating the need to obtain very high levels of expression of the transgene to achieve therapeutic effects.

METHODS FOR GENE TRANSFER

A variety of systems have been developed for gene transfer and expression in mammalian cells. In the case of inherited genetic errors, the choice of a gene delivery system is dictated by the need for persistent expression of the therapeutic gene. This can be most readily achieved if the exogenous genetic material is stably integrated into the host cell genome, thus ensuring the transmission of the transferred gene to the progeny of that cell during normal cell division. In the absence of integration, the inserted gene may be diluted out as the cell divides, and thus the therapeutic effects will ultimately be lost. Gene transfer vectors based on integrating viral systems such as gammaretroviruses, lentiviruses, and spumaviruses have been widely used for preclinical and clinical applications of gene therapy for PIDs.

VECTORS BASED ON RETROVIRUSES

Retroviral vectors are gene transfer constructs that reproduce the replication and integration mechanisms of retroviruses. The Moloney murine leukemia virus (MoMLV) of the mammalian type C retroviruses has been the most extensively studied retrovirus model (Miller, 1992), although other viruses from this group have also been exploited. More recently, vectors based on the two other major groups of retroviruses, lentiviruses and spumaviruses, have also become widely used.

MoMLV Vectors

A complete, wild-type MoMLV particle is composed of two copies of the viral RNA contained within a protein capsid and an external phospholipid envelope. Important enzymes, such as the retroviral reverse transcriptase, integrase, and protease, are also packaged within the virion.

The retrovirus life cycle is constituted by (1) binding of the retroviral envelope to a specific receptor on the cell membrane followed by internalization of the viral capsid; (2) uncoating and reverse transcription of viral genomic RNA to produce double-stranded viral DNA; (3) migration through the cytoplasm to the nucleus; and (4) integration of the proviral DNA into the host cell genome. Integration occurs, however, only if the targeted cells are actively proliferating, presumably because nuclear membranes must be disrupted for nuclear DNA transport. After reverse transcription the viral genome (provirus) consists of 8.3 kb of double-stranded DNA containing the *gag*, *pol*, and *env* genes flanked by the viral long terminal repeats (LTRs). The *gag* and *env* genes encode the structural proteins of viral core and envelope, respectively, while the reverse transcriptase, integrase, and protease are derived from the *pol* region. The two identical LTRs contain sequences promoting and enhancing transcription, as well as a polyadenylation signal (polyA). Between the 5′ LTR and the *gag* gene is a region of the genome (termed Ψ) containing the encapsidation

Table 61.1 PRESENT STATUS AND CHALLENGES OF GENE THERAPY APPROACHES FOR IMMUNODEFICIENCY DISEASES

DISEASE	EFFICACY OF BMT	POTENTIAL TARGET CELLS FOR GENE TRANSFER	STATUS OF CORRECTIVE GENE TRANSFER STUDIES	CHALLENGES	AVAILABLE ANIMAL MODEL
ADA-SCID	Good (HLA-identical)	BM, CB, T	Ongoing clinical trials	Adequacy of immune repertoire in T-cell–directed protocols	muKO
CGD	Poor	BM, CB	Ongoing clinical trial	No selective advantage	muKO
LAD I	Good	BM, CB	Prior clinical experience; current preclinical	Tissue-specific gene expression, no selective advantage	muKO
X-SCID	Good	BM, CB	Ongoing clinical trials	Definition of risk of insertional oncogenesis	Dog, muKO
JAK3-SCID	Good	BM, CB	Prior clinical experience	Definition of risk of insertional oncogenesis	muKO
WAS	Good (HLA-identical)	BM, CB	Ongoing clinical trials	Coexistence of gene-corrected and residual mutated cells may be problematic.	muKO
PNP deficiency	Poor*	BM, CB, T	Approved clinical trial	Adequacy of immune repertoire in T-cell–directed protocols	muKO
ZAP70-SCID	Good*	BM, CB	Preclinical	Lineage/differentiation stage restricted expression	muKO
MHC class II deficiency	Poor	BM, CB	Preclinical	Regulated gene expression	muKO
X-HIM	Fair	BM, CB, T	Preclinical	Regulated gene expression, no selective advantage	muKO
XLA	Limited experience	BM, CB	Preclinical	Restricted gene expression	Xid mouse, muKO
RAG-1	Good	BM, CB	Preclinical	Balance efficacy/toxicity	muKO
RAG-2	Good	BM, CB	Preclinical	High levels of gene expression may be necessary.	muKO
CD3γ deficiency	Good*	BM, CB, T	Preclinical	Regulated gene expression	muKO
Artemis deficiency	Fair	BM, CB	Preclinical	Toxicity of ectopic expression?	muKO
Reticular dysgenesis	Poor	BM, CB	Preclinical	Need for expression in myeloid lineages	zfMO

*Only few cases reported.

BM, bone marrow progenitors; CB, cord blood progenitors; T, peripheral T lymphocytes; muKO, murine knockout model; zfMO, zebrafish morpholino knockdown model.

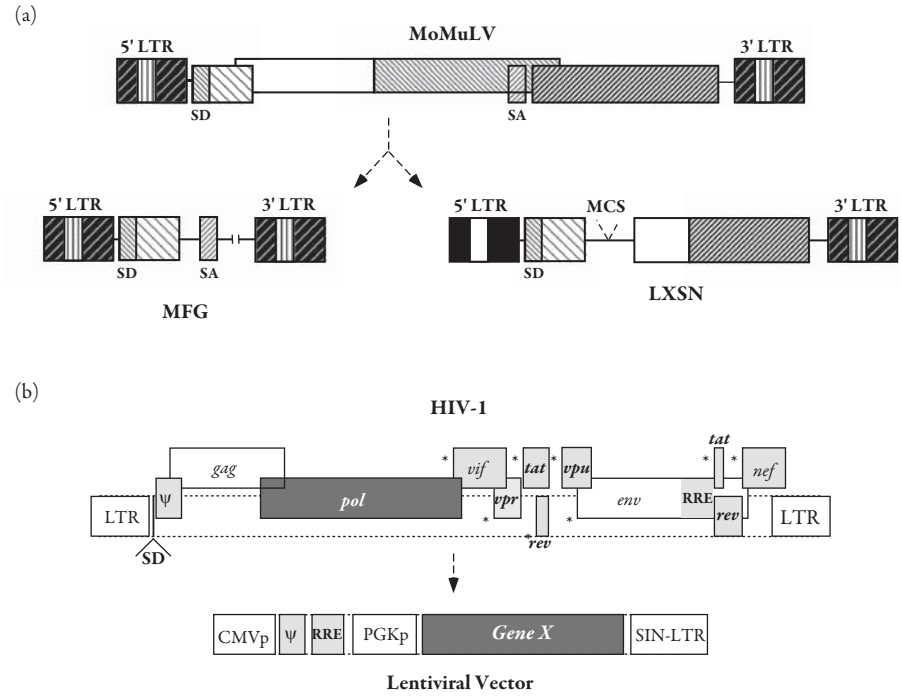

Figure 61.1 (A) Schematic representation of the Moloney leukemia virus (MoMuLV) and MoMuLV-based LXSN (244) and MFG (245) retroviral vectors. The organization of MoMuLV *gag*, *pol*, and *env* genes, as well as of splice donor (SD) and acceptor (SA) sites, packaging signal sequence (ψ), and long tandem repeats (LTRs) is represented. The MoMuLV *gag*, *pol*, and *env* sequences are deleted to obtain the backbone of retroviral vectors. The MFG cassette is an example of a "splicing vector" and maintains both splice donor and acceptor sites of the MoMuLV *env* coding sequence, thus mimicking the natural pattern of viral mRNA splicing. In the LXSN cassette and the MoMuLV 5′ LTR is replaced with the homologous region from the Moloney murine sarcoma virus (MoMuSV). MCS, multiple cloning site; *neo*, neomycin resistance gene; SV40p, SV40 early promoter region. (B) Schematic representation of HIV-1 and of a general lentiviral vector. The organization of HIV-1 *gag*, *pol*, and *env* genes, as well as of the *vif*, *vpr*, *rev*, *vpu*, and *nef* accessory genes and of *tat* and RRE regulatory sequences, is shown. The splice donor (SD) site is indicated and the alternative splice acceptor sites are marked with asterisks. The packaging signal sequence (ψ) and long tandem repeats (LTRs) are represented. Only the packaging signal, RRE region, and 3′ LTR are conserved in the lentiviral vector construct. CMVp, cytomegalovirus promoter; PGKp, phosphoglycerate kinase promoter; SIN, self-inactivating.

sequence that is necessary for efficient packaging of the viral genomic RNA into the capsidic structure (Temin, 1984).

To obtain viral vectors incapable of replicating in the transduced cells, the genes encoding viral proteins are deleted and replaced with selectable markers and/or multiple cloning sites for the insertion of experimental genes (Fig. 61.1). To obtain complete and infectious viral particles, "packaging" cell lines have been developed that express the *gag*, *pol*, and *env* gene products but are unable to encapsidate the viral genomic RNA because of mutations or deletions in their Ψ region. However, these cells allow packaging of the genome of retroviral vectors in which the Ψ region is conserved (Fig. 61.2).

Several packaging cells are available that contain and express different *env* genes, which affords them different host ranges. Among these, the PG13 (Miller et al., 1991) and the FlyRD18 (Cosset et al., 1995) packaging lines incorporate the gibbon-ape leukemia virus (GALV) and the ct endogenous virus (CEV) envelope proteins, respectively. In hematopoietic cells and progenitors, the expression levels of receptors for GALV and CEV are higher than the receptor for the Moloney envelope, and in some cases, receptors can be metabolically upregulated (Bunnell et al., 1995). These characteristics make these packaging cells effective tools for diseases potentially treatable by gene transfer into peripheral blood elements or HSCs (Kelly et al., 2000; Kiem et al., 1998).

Another packaging system is based on the incorporation of the vesicular-stomatitis virus envelope protein G (VSV-G) into the virions of MoMLV-derived vectors. The particles carrying the VSV-G have the advantage of a broader host range compared to virions encapsidated into the amphotropic envelope protein and allow the generation of concentrated, high-titer retroviral vector preparations (Rebel et al., 1999).

In vitro gene transfer is usually achieved by single or repeated exposure of the target cells to retroviral supernatants. Alternative procedures involve cocultivation of retroviral producer and target cells, which can increase gene transfer efficiency but pose significant hurdles to the development of clinical protocols.

One advantage of using retroviral vectors for gene transfer is the broad range of infectable cell types and the efficient gene integration that can be achieved with dividing cells. Retroviral-based vectors, however, also present drawbacks. The maximum amount of DNA that these vectors can accommodate is limited to about 8 kb, a size that prevents the transfer of large genomic sequences. Furthermore, retroviral vectors integrate randomly in the host cell genome, raising a twofold problem.

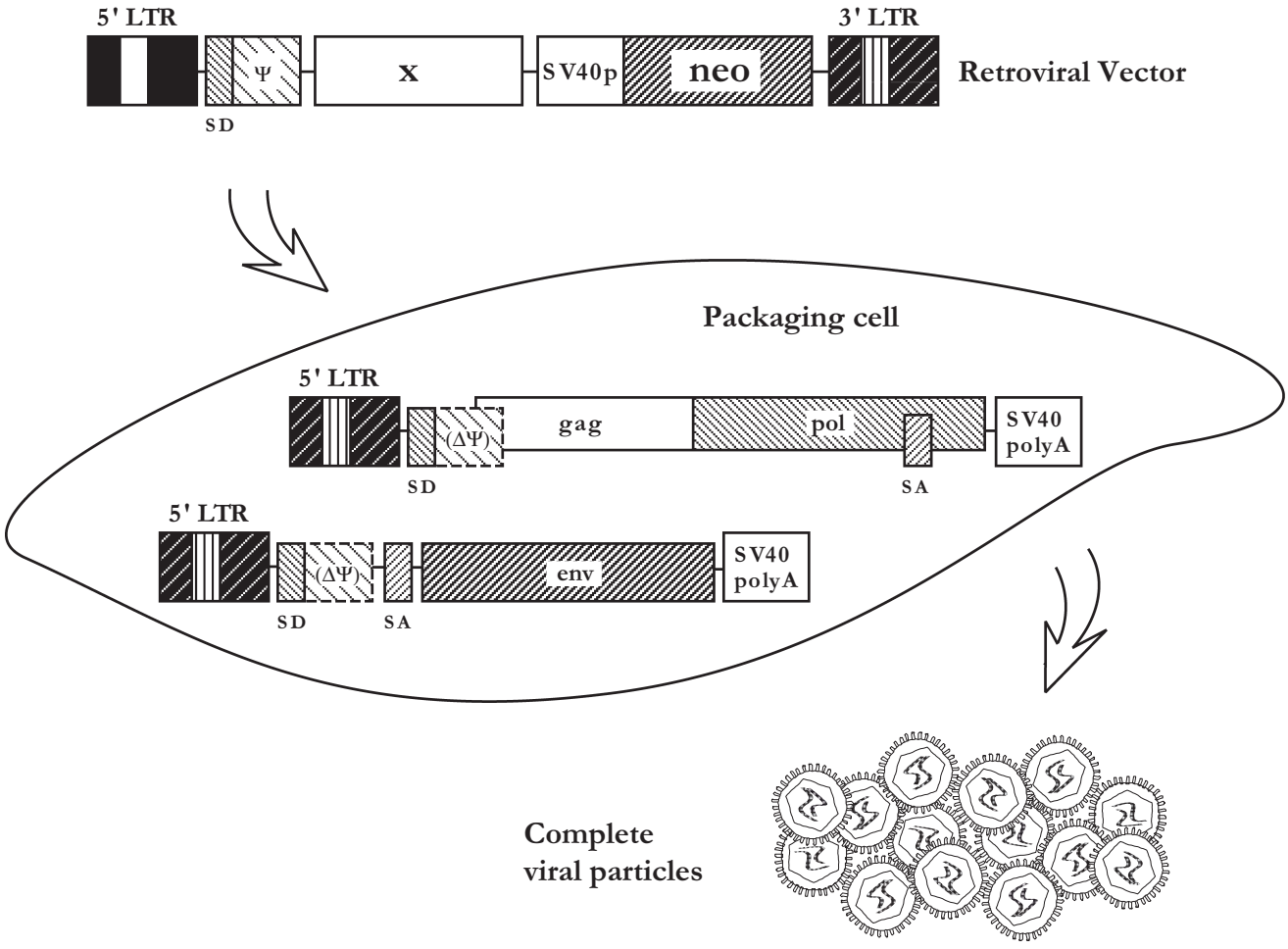

Figure 61.2 Production of retroviral vector supernatants. The retroviral construct is transfected into the packaging cell line that contains the "helper" MoMuLV modified genomes. The *gag*, *pol*, and *env* genes provide the necessary viral proteins, whereas deletions of the packaging signal sequences prevent the encapsidation of the "helper" genomic RNA into virions. The retroviral vector, however, contains the complete packaging signal and generates RNA molecules that can be efficiently encapsidated. Finally, infectious viral particles containing the recombinant retroviral vector can be obtained in the culture supernatant. Lentiviral vector production is achieved following similar procedures. However, in most cases, transient cotransfection of the lentiviral vector and helper HIV-1 sequences is performed.

First, the levels of gene expression can drastically vary from cell to cell depending on the particular site of proviral integration. These "position effects" are thought to depend on the basal chromatin structure at the integration site and can completely silence transgene expression in some cases while augmenting expression in others. Clearly, many genes operate within tightly regulated ranges of expression, and this wide variability is not acceptable. Second, the random integration events could theoretically disrupt critical genetic sequences leading to cell death or activation of oncogenes, resulting in malignancy. The risk of insertional oncogenesis is unfortunately real, as recently indicated by the development of a leukemia-like disorder in subjects treated with retroviral-mediated gene transfer as a treatment for X-SCID (Hacein-Bey-Abina et al., 2008; Howe et al., 2008) and WAS (Braun et al., 2011).

Lentiviral Vectors

Gene transfer vectors based on lentiviruses such as HIV-1 have advantages over retroviral vectors for transduction into quiescent lymphocytes and HSCs. The genome of HIV-1 is more complex than that of other retroviruses. In addition to the structural *gag*, *pol*, and *env* genes, it also contains two regulatory genes, *tat* and *rev*, and four additional transcripts, *vif*, *vpr*, *vpu*, and *nef*, which encode accessory proteins critical for viral replication and pathogenesis.

In contrast to type C retroviruses, the replicative cycle of lentiviruses is independent of cell division and dissolution of the nuclear envelope during cell mitosis. Lentivirus vectors are therefore potentially able to integrate into the genome of nondividing host cells. A number of gene transfer systems based on HIV-1, HIV-2, and non-primate lentiviruses, such as equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV), have been described (Arya et al., 1998; Naldini et al., 1996a; Olsen, 1998; Poeschla E et al., 1998; Poeschla EM et al., 1998; Reiser et al., 1996; Uchida et al., 1998). Naldini et al. (1996a) first developed an HIV-1-based gene transfer system in which replication-defective viral particles were obtained by pseudotyping the HIV-1 core—that is, packaging it into heterologous envelope of the MoMLV or the

VSV-G protein. This strategy not only increased the safety of the system by excluding the HIV envelope but also broadened the tropism of the vectors to allow transduction of many cell types. Several studies have shown that lentiviral vectors can efficiently transduce nondividing cells in experimental conditions (Case et al., 1999; Kafri et al., 1997; Miyoshi et al., 1997, 1999; Naldini et al., 1996b; Poeschla E et al., 1998; Poeschla EM et al., 1998; Uchida et al., 1998; Zufferey et al., 1997) and have affirmed this class of vectors as useful tools for gene therapy of diseases affecting tissues composed of quiescent or postmitotic cells, such as neuronal and retinal cells, hepatocytes, myocytes, and lymphohematopoietic cells.

Obvious biosafety concerns have been raised for vectors derived from HIV-1, a highly pathogenic human virus. These included the worry that unwanted recombination events during vector production could generate replication-competent viruses. If this occurred, patients receiving gene therapy could be at risk for infection by an HIV-like virus. In addition, treated patients carrying integrated HIV sequences might subsequently come in contact with wild-type HIV, in which case recombination between virus sequences in vivo could mobilize the gene therapy vector, leading to spread of a new virus to additional patient cells or even to infection of individuals in contact with the patient (Evans & Garcia, 2000). Systems have been generated that use nonoverlapping split-genome packaging constructs and self-inactivating vector cassettes to decrease these risks (Dull et al., 1998; Miyoshi et al., 1998; Zufferey et al., 1998).

On the other hand, mapping studies of lentiviral vector integration sites indicate that these vectors may have less tendency to target dangerous transcription start sites than vectors based on MLV (Biffi et al., 2011; Hematti et al., 2004; Ronen et al., 2011; Wu et al., 2003), thus supporting the notion that these vectors may be less prone to induce insertional oncogenesis.

Foamy Virus Vectors

Foamy viruses (FV) are nonpathogenic retroviruses that are commonly found in mammalian species. In contrast with other retroviruses, reverse transcription in FV occurs during virion formation, and infectious viral particles therefore contain DNA genomes. This characteristic may improve transduction of quiescent cells, in which inefficient reverse transcription can prevent transduction by oncoviral or lentiviral vectors. This and other favorable characteristics, including broad tropism, large genome size (>13 kb), and lack of pathogenicity, have stimulated their development as gene transfer vectors. The FV genome contains the *gag*, *pol*, and *env* genes, but also three additional genes (*bel-1*, *bel-2*, *bel-3*) located between *env* and the 3′ LTR and expressed from an internal viral promoter. The *bel-1* gene encodes for Tas, a transcriptional transactivator of the FV LTR and internal promoter. In the absence of Tas, the FV LTR is silent. The function of the *bel-2* and *bel-3* genes is unknown. The FV Gag protein contains a functional nuclear localization signal, raising the possibility that nuclear entry of FV genomes is independent of mitosis (Rethwilm, 1995).

In the past 10 years, replication-defective FV vectors have been generated and perfected. The initial version of the vectors carried deletions that abolish *bel-1* expression and contain expression cassettes (composed on an internal promoter and the gene of interest) within the two FV LTRs. More recent constructs have reduced the FV sequences to a minimum, making abundant space (>9 kb) for a transgene (Park et al., 2002; Russell & Miller, 1996; Trobridge et al., 2002; Wu et al., 1998; Wu & Mergia, 1999). Transient expression systems or packaging cells provide the structural and regulatory FV gene products and allow for production of complete, infectious viral particles (Russell & Miller, 1996; Trobridge et al., 2002; Wu & Mergia, 1999). The virions are stable, are not inactivated by human serum (Wu et al., 1998), and can be concentrated by ultracentrifugation to titers of about 10^7/mL (Trobridge et al., 2002).

FV vectors have been shown to efficiently enter quiescent cells where functional vector genomes survive until these cells are stimulated to divide, suggesting that, similar to HIV vectors, a stable intracellular transduction intermediate is formed (Mergia et al., 2001; Russell & Miller, 1996). This property may account for the ability of FV vectors to efficiently transduce hematopoietic cells, including murine and human pluripotent HSCs (Josephson et al., 2002; Trobridge et al., 2002; Vassilopoulos et al., 2001; Zucali et al., 2002), which has supported their use to correct animal models of hematopoietic diseases, such as Fanconi anemia, LAD type 1, and WAS (Bauer et al., 2011; Si et al., 2008; Uchiyama et al., 2012).

RESULTS OF GENE THERAPY TRIALS FOR IMMUNODEFICIENCIES

ADA DEFICIENCY

Genetic defects resulting in loss or severe reduction of ADA (MIM *608958, #102700; EC 3.5.4.4) result in extreme reduction of lymphocyte numbers and impairment of immune functions (Hirschhorn, 1993; Markert, 1994) (see Chapter 14). Complete ADA deficiency causes SCID (ADA-SCID).

In the mid-1980s, ADA deficiency was identified as the initial candidate disorder for trials of gene therapy because of a combination of favorable characteristics. The organization of the ADA gene and cDNA was well known, and ADA gene function and regulation had been clearly defined (Orkin et al., 1983; Valerio et al., 1983; Wiginton et al., 1983). A wide array of enzyme concentrations, ranging from 10 percent of normal to about 50 times the normal mean, was known to be compatible with normal immunity (Daddona et al., 1983; Valentine et al., 1977). This situation provided an ideal scenario where the expression of the transferred gene was not required to be tissue-specific or tightly regulated. In addition, the experience from allogeneic hematopoietic stem cell transplantation (HSCT) experiments suggested that normal ADA-expressing cells had a selective advantage over the ADA-deficient bone marrow progenitors, thus eliminating the need for myeloablation preceding the infusion of the patient gene-corrected HSCs. Finally, the fact that some HSCT patients achieving

engraftment of only donor T cells were shown to have full reconstitution of lymphocyte functions provided the rationale for the correction and reinfusion of patient mature T lymphocytes as an alternative, and technically less complex, gene therapy approach.

The first clinical gene therapy protocol for ADA deficiency was begun on September 14, 1990, at the National Institutes of Health (NIH). It enrolled two ADA-deficient girls with persisting signs of immunodeficiency despite the institution and regular administration of polyethylene glycol-ADA (PEG-ADA) replacement therapy. This protocol involved apheresis of peripheral blood and culture of each patient's peripheral blood mononuclear cells with IL-2 and anti-CD3 antibody to stimulate intense proliferation of the small number of T lymphocytes from each patient's circulation. Once actively proliferating, cells were exposed to supernatants containing the LASN retroviral vector expressing the human ADA cDNA and expanded about 100-fold before being reinfused into the patient (Tuschong et al., 2002). Over a 2-year period, both patients underwent a series of treatments with the intention to target, and thus correct, a large proportion of each patient's T-cell repertoire. Both children improved or developed de novo several humoral and cellular immune functions, including the ability to produce IgM in vitro upon stimulation of cultured cells with pokeweed mitogen (PWM), improved in vivo antibody responses to vaccines against *Haemophilus influenzae* type B (HIB) and tetanus toxoid, normalization of isohemoagglutinin titers and T-cell counts, development of cutaneous delayed-type hypersensitivity to various antigens, normalization of T-cell production of IL-2 after stimulation with specific antigens (tetanus toxoid and influenza A virus), and development of in vitro cytolytic T-cell–specific activity against allogeneic cells and influenza A virus-infected autologous cells. All these immune responses were absent or barely detectable before gene therapy (Blaese et al., 1995).

Ten years after the last cell infusion, about 20 percent of circulating T cells from the first patient still contained the ADA vector and expressed the transgene, while less than 0.1 percent of T cells from the second patient had detectable vector sequences. Both patients continue to receive weekly injections of PEG-ADA, although at a reduced dose. Numbers of peripheral blood CD3+ T cells remained in the normal range for 7 years even as the dose of PEG-ADA was lowered by 50 to 60 percent, but more recently showed signs of decline, although their proliferative responses to mitogens and recall antigens have remained at the lower range for healthy controls.

The marked difference between the two patients in the achieved level of gene marking and ADA expression appears to be the result of several factors. In vitro proliferation of cells from the second patient was poor, limiting the transduction efficiency. In addition, this patient developed a persistent immune response against the retroviral envelope and the lipoprotein components of the fetal calf serum used to culture the cells, and this response may have induced immune-mediated elimination of infused cells (Muul et al., 2003; Tuschong et al., 2002).

The long-term clinical benefit of this first gene therapy trial is difficult to evaluate because of the concomitant administration of PEG-ADA. Nevertheless, several unique pieces of information could be drawn from this study, including the safety of administering genetically modified autologous T cells, the long persistence (>12 years) of such cells in vivo, and the long-term transgene expression that can be obtained in vivo by the MoMLV retroviral promoter (Muul et al., 2003). In addition, the results of this trial point out that immune responses, even in immunodeficient patients, can confound the outcome of gene therapy experiments in unpredicted ways (Muul et al., 2003; Tuschong et al., 2002).

Following protocol and vector preparations identical to those used in the NIH trial, in August 1995 Dr. Sakiyama and colleagues from Hokkaido University School of Medicine in Sapporo treated a Japanese ADA-SCID patient with 11 infusions of genetically modified autologous T lymphocytes over a period of 20 months. Periodic analysis of peripheral blood lymphocytes showed transduction frequencies of 10 to 20 percent (Egashira et al., 1998; Onodera et al., 1998), accompanied by gradual increases of ADA enzyme activity in the patient's circulating T cells to levels comparable to those of a heterozygous carrier. Evidence of improvement of the patient's immune function after gene therapy included increased T-lymphocyte counts and delayed-type hypersensitivity responses, appearance of isohemagglutinins, increase in serum immune globulin, and specific antibody production (Egashira et al., 1998; Kawamura et al., 1999; Onodera et al., 1998). Similarly to the first patient in the NIH trial, follow-up studies demonstrated long-term persistence of transduced and polyclonal T cells (Misaki et al., 2001).

A second gene therapy clinical trial for ADA deficiency began in 1992 in Milan, Italy; Bordignon and coworkers used two distinguishable forms of the same retroviral vector to transduce separately lymphocytes and bone marrow cells from two ADA-deficient patients who had showed a progressive decline of efficacy of PEG-ADA therapy. This study involved the in vitro culture, retroviral transduction, and reinfusion of patients' lymphocytes and bone marrow cells and aimed to define the specific contribution of gene-corrected mature T lymphocytes and bone marrow progenitors to long-term immune reconstitution (Bordignon et al., 1993). The two patients were treated with intravenous infusions of autologous transduced cells over a period of 9 to 24 months, during which PEG-ADA dosage was decreased. Normalization of lymphocytes counts and isohemoagglutinin titers, restoration of antigen-specific antibody production, and mitogen- as well as antigen-specific T-cell proliferation were observed in both patients. At the molecular level, both forms of the integrated provirus were individually detectable in the patients' peripheral lymphocytes, with the clear long-term predominance of the construct used to transduce bone marrow cells. Of particular interest, vector-derived ADA activity was detectable in bone marrow cells, peripheral lymphocytes, and also in mature granulocytes and erythrocytes, indicating that the correction of bone marrow progenitors was stably transferred to their progeny (Bordignon et al., 1995).

Similar to the previous U.S. study, the clinical impact of gene therapy in these patients was difficult to assess because of concomitant administration of PEG-ADA, which on the one

hand might have contributed to the immunological improvement, but on the other may also have abolished the potential selective advantage of genetically engineered cells over the defective cell populations in ADA-deficient patients. One of the patients treated in Italy with multiple infusions of transduced peripheral blood lymphocytes offered the opportunity to evaluate the latter hypothesis directly when complications attributed to the PEG-ADA treatment developed that justified the discontinuation of the enzyme injections. Withdrawal of PEG-ADA led to a selective growth advantage of gene-transduced and ADA-expressing circulating T lymphocytes that from a prevalence of about 3 percent eventually reached nearly 100 percent. Absolute $CD3^+$ T-cell counts also rose to levels significantly higher than those observed during PEG-ADA treatment, and intracellular ADA activity increased to levels of approximately 50 percent of normal individuals. These findings were accompanied by full restoration of T-cell function that had been inadequate during PEG-ADA treatment. The increase of lymphocyte ADA activity, however, was not sufficient to achieve systemic detoxification; red blood cell deoxyribonucleotides (dAXP) increased to levels substantially higher than those observed in ADA-deficient patients treated with BMT (Aiuti et al., 2002b). These findings suggested that even high numbers of genetically corrected lymphocytes were insufficient for achieving full, lasting metabolic correction of ADA deficiency and justified additional attempts by the same investigators at correcting the HSC as a more efficient approach for gene therapy of this disease.

Following up on these findings, and reasoning that an initial developmental advantage was important for the transduced HSC, the Italian investigators decided to create space in the bone marrow by administering a low dose of busulfan (2 mg/kg/day for 2 days before infusion of gene-corrected cells) to a series of 10 ADA-deficient patients before the infusion of gene-corrected cells. This approach revealed extremely effective: nine patients showed immune reconstitution with increases in T-cell counts and normalization of T-cell function. In five patients, B-cell function was restored as assessed by antigen-specific antibody responses after vaccination. Eight patients have remained off PEG-ADA therapy in the long term. The degree of myelosuppression was generally mild and resulted in prolonged neutropenia and thrombocytopenia only in two patients. Importantly, ADA enzymatic activity was documented in blood mononuclear cells and red cells at levels significantly higher than at baseline and resulted in significant reduction of toxic levels of deoxyadenosine nucleotides in the red cells.

The presence of the retroviral ADA gene was detected in bone marrow (in 5 percent of $CD34^+$ cells) and peripheral blood specimens (50–90 percent of T cells, B cells, and natural killer [NK] cells). The fraction of gene-corrected CD34+ cells found in patients at 1 year after treatment correlated with the dose of infused gene-corrected $CD34^+$ cells and the actual response to the myelosuppressive agent. All patients were alive and there were no adverse events due to the retrovirally transduced cells. PEG-ADA was initiated or restarted in two patients, due to insufficient efficacy of the procedure (Aiuti et al., 2002a, 2009). Because in this trial, in addition to receiving chemotherapy, patients also were not given PEG-ADA, it remains to be established what variable had the major impact on the outcome of the procedure.

These very exciting results marked a clear difference from those obtained from previous clinical gene transfer attempts into the HSC of ADA-deficient patients that were carried out in the mid-1990s in the United States and Europe that did not include either the use of chemotherapy or the withdrawal of PEG-ADA. One such trial had been initiated by Kohn and collaborators in 1993 at the Children's Hospital Los Angeles. Notably, this was the first gene therapy protocol utilizing umbilical cord blood $CD34^+$ hematopoietic progenitors. Three newborns were enrolled in this protocol, which involved separation of $CD34^+$ progenitors from the cord blood immediately after birth, repeated retroviral transductions with the normal ADA cDNA, and intravenous infusions into the patients on their fourth day of life. PEG-ADA treatment was also instituted and maintained in all three patients. Serial molecular studies demonstrated that integrated vector sequences were detectable in bone marrow-derived $CD34^+$ cells, in granulocyte-macrophage colony-forming units (CFU-GM), and in both the mononuclear and polymorphonuclear mature leukocyte populations of all three infants (Kohn et al., 1995).

Four years after the infusion of gene-corrected cells, 1 to 10 percent of circulating T lymphocytes contained the transduced gene, whereas only 0.01 to 0.1 percent of other hematopoietic elements were vector-positive. After PEG-ADA treatment was stopped in the patient carrying the highest percentage of gene-corrected T lymphocytes (around 10 percent), the proliferative response to tetanus toxoid was lost, as were most of circulating B and NK cells. Reduction of total numbers of $CD3^+$ and $CD4^+$ T lymphocytes was also observed; however, the percentage of circulating T cells containing the transduced ADA gene increased to 30 to 100 percent, and lymphocyte proliferation response to PHA was retained. These findings and the development of an upper respiratory tract infection induced the investigators to reinstate PEG-ADA therapy, leading to restoration of clinical and laboratory findings to prewithdrawal levels. The low levels of ADA gene marking and expression did not justify the suspension of PEG-ADA treatment in the other patients (Kohn et al., 1998).

These observations supported the notion that ADA gene-corrected T cells have a selective survival advantage over unmodified lymphocytes. However, the trial also demonstrated that the level of transduced progenitors (particularly for B and NK cells) was not sufficiently high to guarantee sustained, adequate immune function in the absence of enzyme replacement. A possible contributing reason for these findings was that the expression of ADA afforded by the LASN vector in HSCs was not adequate to allow differentiation and function of large numbers of mature lymphocytes. Based on this last hypothesis, a two-site trial using ADA vectors with higher expression capabilities was started in 2001 at Children's Hospital Los Angeles and NIH with the aim of comparing the relative efficacy of two different vectors in vivo after transduction in bone marrow $CD34^+$. In the first phase of this trial, patients were maintained on

PEG-ADA treatment and no chemotherapy was administered. Long-term, low-level marking was achieved in two of four treated patients, but no immunological improvement was obtained. In its second phase, the trial involved low-dose busulfan chemotherapy and withdrawal of PEG-ADA treatment. Six patients were enrolled, with three achieving adequate immune reconstitution off PEG-ADA. These results demonstrated that the use of reduced-intensity conditioning improved the level of engraftment of gene-modified stem cells and the subsequent production of ADA-expressing lymphocytes, contributing to immune reconstitution. In addition, the use of two retroviral vectors in each patient in this trial indicated the superiority of a vector carrying backbone modifications aimed to reduce methylation and silencing in vivo (Candotti et al., 2012).

An additional French and British pilot trial of gene therapy for ADA deficiency in 1993 involved retroviral transduction of HSCs isolated from bone marrow (Hoogerbrugge et al., 1996). Three ADA-deficient children were enrolled. The results were disappointing, since vector-containing cells were detectable by polymerase chain reaction (PCR) 6 months after the treatment in the bone marrow of only one patient, and no signal was detected in the peripheral blood of any of the three children. No clinical improvement was observed in these patients. Based on their preclinical results in the Rhesus model, the authors theorized the need for preemptive myeloablation to obtain efficient engraftment of gene-corrected stem cells in ADA deficiency. Following this hypothesis, a more recent British trial enrolled six patients who were treated following withdrawal of PEG-ADA and administration of nonmyeloablative chemotherapy with melphalan (140 mg/m^2) or busulfan (4 mg/kg). In two of these patients, PEG-ADA was restarted due to absence of measurable gene marking in peripheral blood cells. The other patients showed increase in T-cell and B-cell numbers, with normalization of lymphocyte responses to PHA and anti-CD3 and adequate immunoglobulin production in three patients. Significant reduction of deoxyadenosine nucleotide levels was observed after gene therapy in the patients who were able to discontinue PEG-ADA (Gaspar et al., 2006, 2011a).

Based on cumulative data from more than 40 ADA-SCID patients treated with gene therapy, it appears that selective advantage of gene-corrected cells is not sufficient to provide immune reconstitution in the absence of preparative chemotherapy. This notion is also supported by the results of the Japanese clinical trial that was started in 2003 with the enrollment of two patients who received gene-corrected CD34$^+$ bone marrow cells after discontinuation of PEG-ADA, but without chemotherapy. Recent results from this trial indicate that full immune reconstitution was not achieved in the absence of conditioning (Otsu et al., 2010).

In summary, while BMT from an HLA-identical sibling remains the therapy of choice for ADA deficiency, the results obtained by the pioneering and current clinical trials have indicated that gene therapy approaches can be effective and to date maintain a record of safety. Indeed, it can be argued that gene therapy should be considered as the first treatment option in the absence of a matched family donor.

CHRONIC GRANULOMATOUS DISEASE

Genetic defects involving gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}, have been identified in CGD patients who, because of impaired superoxide production in phagocytic cells, usually present early in life with abscesses and/or granuloma formations in the skin, liver, lungs, or bone due to catalase-positive bacteria and fungal pathogens (see Chapter 52). Current management of CGD patients includes antibiotic prophylaxis, administration of IFN-γ, and allogeneic HSCT (Kang EM et al., 2011). For several patients, however, available therapeutic options remain unsatisfactory, and cloning of the genes encoding all the elements of the NAPDH oxidase system (Forehand & Johnston, 1994; Roos, 1994) has allowed gene therapy strategies to be developed.

Several groups have demonstrated the feasibility of viral vector gene transfer in reconstituting the respiratory burst activity of B-cell lines and monocytes from CGD patients (Li et al., 1994; Sekhsaria et al., 1993; Thrasher et al., 1992; Thrasher, Casimir et al., 1995; Thrasher, de Alwis et al., 1995). The ultimate target for gene correction of CGD, however, is the HSC because of the very limited lifespan and the reduced proliferative state of mature phagocytes.

In vitro preclinical studies have demonstrated that CD34$^+$ hematopoietic progenitors derived from peripheral blood after granulocyte colony-stimulating factor (G-CSF) mobilization and genetically corrected by retroviral-mediated gene transfer can show restoration of superoxide production when induced to differentiate into mature monocytes and neutrophils (Becker et al., 1998; Li et al., 1994; Porter et al., 1996; Sekhsaria et al., 1993; Weil et al., 1997). In addition, in vivo restoration of respiratory burst in p47^{phox} and gp91^{phox} knockout mice was demonstrated following gene correction of bone marrow cells with retroviral vectors (Bjorgvinsdottir et al., 1997; Mardiney et al., 1997). On the basis of these encouraging results, Malech and coworkers at the NIH initiated a phase I clinical study for gene therapy of the autosomal recessive form of CGD due to defective expression of the NAPDH complex p47^{phox} subunit. Five CGD patients were subjected to pharmacological mobilization of CD34+ hematopoietic progenitors that were then collected from the peripheral blood by apheresis. Enriched CD34$^+$ cells were transduced in vitro using a retroviral vector expressing cDNA encoding p47^{phox} and then reinfused into the patients without myelosuppression. The appearance of NAPDH oxidase activity in phorbol myristate acetate (PMA)-stimulated peripheral blood granulocytes was followed in each subject over time. At variable intervals between 15 and 32 days after infusion of transduced progenitors, all patients showed oxidase-positive granulocytes, with maximum percentages ranging between 0.004 percent and 0.05 percent. The presence of the transduced gene in the DNA of peripheral blood lymphocytes was confirmed by PCR detection. Oxidase-positive cells were detected for a minimum of 79 and a maximum of 172 days (Malech et al., 1996). Although silencing of the transduced gene has not been excluded, these findings are consistent with loss of the transduced cells and with the possibility that more differentiated progenitors, rather than true self-renewing HSCs, were the primary transduction target in this study.

The same research group conducted a second clinical trial aimed at correction of the more common X-linked form of CGD (X-CGD), caused by mutation of the gp^{91phox} subunit of the NAPDH oxidase. Using a high-titer retroviral vector and improved transduction procedures, four X-CGD patients each received two to four infusions of gp^{91phox}-transduced $CD34^+$ cells. Despite these modifications, the levels of NAPDH-positive neutrophils observed in treated patients ranged between 0.2 and 0.06 percent and persisted for only 6 to 14 months (Malech et al., 1998, 2000).

Similar results were obtained by Dinauer et al. in another phase I trial for X-CGD at the University of Indiana Medical School, where two subjects were treated using mobilized peripheral blood $CD34^+$ cells transduced with a murine stem cell virus–based retroviral vector. In this case, 0.007 to 0.05 percent NAPDH-positive neutrophils were detected in both patients for a period of about 9 months (Goebel & Dinauer, 2003).

The results of these early gene transfer studies for CGD indicated the need for modifications of procedures aimed at increasing efficacy and opened the way to the next series of clinical trials that introduced nonmyeloablative conditioning.

The NIH group administered low-dose busulfan (10 mg/kg) prior to infusion of retrovirally transduced mobilized $CD34^+$ cells in three X-CGD patients with refractory infections. Functionally corrected cells were detected in the peripheral blood of two patients up to levels of 4 to 24 percent of neutrophils, which was associated with complete or partial resolution of the infections. However, corrected cells remained detectable in the long term at levels of around 1 percent in only one patient (Kang EM et al., 2011).

In January 2004, a new trial in Germany by Grez and collaborators used a retroviral vector expressing $gp91^{phox}$ under the transcriptional control of the spleen focus-forming virus LTR to target G-CSF–mobilized $CD34^+$ progenitor cells of two patients with X-CGD. Before the infusion of gene-corrected cells, patients were given intravenous busulfan (4 mg/kg for 2 consecutive days), which resulted in transitory myelodepression from which patients recovered by day 30 after treatment. Around 15 percent of neutrophils were found to be NAPDH-positive early after treatment. This fraction increased due to insertional activation of the *PRDM16* and *MDS1/EVI1* genes in clonal cell populations that expanded with time. However, epigenetic inactivation of the vector gradually reduced the NAPDH-positive neutrophils to less than 5 percent by 1 to 2 years after treatment. Unfortunately, activation of the EVI1 due to retroviral insertion resulted in myelodysplasia with monosomy 7 in both treated patients (Evans & Garcia, 2000). The first patient suffered from septic shock in the context of myelodysplastic syndrome (MDS) and died 2.5 years after gene therapy. The second subject was treated with allogeneic stem cell transplantation and died of complications (Ott et al., 2004; Stein et al., 2010; Aiuti et al., 2012).

The same protocol was used in Switzerland by Seger and collaborators, who enrolled a X-CGD boy with severe *Aspergillus* infection. NAPDH-positive neutrophils were detected at levels up to 30 percent, and the procedure resulted in eradication of the fungal infection (Bianchi et al., 2009). Unfortunately, also this patient developed MDS and has undergone allogeneic stem cell transplantation (Aiuti et al., 2012).

Another gene therapy trial using a Moloney retroviral vector was conducted in South Korea and enrolled two X-CGD patients. Mobilized peripheral blood $CD34^+$ cells were transduced and infused after a conditioning regimen of fludarabine and busulfan. The level of NAPDH-positive cells reached 6 to 14 percent of neutrophils, but the correction was short term and declined to less than 1 percent in both patients 3 years after treatment (Kang HJ et al., 2011).

Additional, unpublished experience of gene therapy for CGD has been generated by Thrasher in London, where conditioning with melphalan (140 mg/m^2) and either the vector employed in the German trial or the NIH construct were used. Similar to other trials, in the short term (<2 months), low levels of $gp91^{phox}$ expression were detected in treated patients (Grez et al., 2011).

In summary, therefore, the current experience with gene therapy of CGD points to a yet unexplained difficulty in achieving long-term engraftment of transduced cells. The lack of a strong selective advantage of gene-corrected populations in this disease may play a major role and may indicate that more significant levels of HSC transduction and engraftment will be needed to obtain clinical benefit.

More efficient methods of gene transfer are also being developed based on lentiviral vectors (Naumann et al., 2007; Roesler et al., 2002; Santilli et al., 2011; Saulnier et al., 2000). Alternatively, in vivo selection of gene-corrected cells could be engineered by the addition of drug resistance markers such as the multidrug resistance (MDR) or dihydrofolate reductase (DHFR) genes to gene transfer vectors. Studies of these approaches in animal models are ongoing and are likely to provide clear indications on the prospects of future success of gene therapy for CGD.

LEUKOCYTE ADHESION DEFICIENCY TYPE I

LAD I is an inherited defect of leukocyte functions caused by the absence or reduced levels of expression of the common β_2-integrin subunit (CD18) (Harlan, 1993). LAD I primarily affects motion functions of granulocytes that fail to migrate into inflamed tissues and exert their phagocytic functions. Affected patients initially present with retarded separation of the umbilical cord and subsequently develop recurrent severe bacterial and fungal infections (Etzioni, 1994; Fischer et al., 1988) (see Chapter 52). Allogeneic BMT from an HLA-matched related donor has been demonstrated to be curative for this disease. The insertion of normal cDNA into the stem cells of a patient may therefore lead to genetic correction of this disease, and studies utilizing viral vectors have demonstrated in vitro functional reconstitution of CD18 molecules in LAD I lymphocytes (Bauer et al., 1995; Hibbs et al., 1990; Wilson et al., 1990).

The expression of CD18 is limited to myeloid cells and lymphocytes; however, functional adhesion molecules require dimerization of CD18 with other subunits (CD11a, CD11b, or CD11c) also expressed only by the same cell types. Expression of CD18 in other lineages deriving from gene-

corrected HSCs and normally not expressing CD18 should therefore not result in unregulated and unwanted expression of complete adhesion molecules.

Similarly to CGD, because of the short lifespan of myeloid cells, the target for corrective gene transfer of LAD I is necessarily the HSC. Retroviral vectors have been used to demonstrate reconstitution of CD18 expression in granulocytes differentiated in vitro from gene-corrected $CD34^+$ cells (Bauer et al., 1998; Yorifuji et al., 1993). Following these studies, a clinical protocol for gene therapy of LAD I at the University of Washington in Seattle used retroviral-mediated correction of G-CSF–mobilized peripheral blood $CD34^+$ cells. Bauer and Hickstein (2000) reported results from the two LAD I patients treated. Peripheral blood $CD34^+$ cells were obtained by pharmacological mobilization with G-CSF and exposed to a retroviral vector expressing CD18 before being reinfused into the patients. One month after the infusion of transduced $CD34^+$ cells, both patients showed low percentages (0.03–0.04 percent) of $CD18^+$ myeloid cells, and one patient had some evidence of functional correction, as $CD18^+$ myeloid cells were demonstrated to migrate into a site of inflammation. Unfortunately, gene-corrected cells were not detectable beyond 2 months after treatment in these two patients (Bauer & Hickstein, 2000).

Allogeneic HSCT studies in a canine model of LAD I have shown that very low levels of donor $CD18^+$ neutrophils (<500/μL) were sufficient to reverse the disease phenotype and that nonmyeloablative conditioning is adequate for correction of the disease phenotype (Bauer et al., 2004, 2005; Sokolic et al., 2005). These experiments were followed by HSC gene-correction experiments in this model using murine retroviral vectors (Bauer et al., 2006), FV vectors (Bauer et al., 2008), and lentiviral vectors (Hunter et al., 2011a, 2001b; Nelson et al., 2010) that, in most cases, showed correction of the canine LAD phenotype. However, no selective survival advantage of gene-corrected myeloid cells is expected in LAD I. As with CGD, strategies involving adequate preparative myeloablation, in vivo selection, and the use of efficient vectors will likely prove useful in improving the outcome of future gene therapy trials for LAD I.

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY

X-SCID is caused by mutations of the common gamma chain (γ_c) of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Noguchi et al., 1993; Puck et al., 1993). Affected males present with a combined impairment of T- and B-cell immunity, despite the presence of B cells in normal or even elevated numbers (see Chapter 10). Allogeneic BMT can be curative for these patients, and it generally shows good success in terms of survival (Antoine et al., 2003; Buckey, 2004) (see Chapter 60). However, especially in the case of haploidentical donors, transplanted patients often achieve only partial chimerism of hematopoietic lineages, with persistence of autologous B lymphocytes and impairment of humoral immune function that requires continuous replacement therapy with immunoglobulins (Antoine et al., 2003). Such unsatisfactory results have fostered the development of gene therapy approaches for this disease.

In preclinical studies, several groups of investigators have shown that retroviral-mediated transfer of the γ_c into B lymphoblastoid cell lines derived from X-SCID patients can correct a series of cellular defects, including γ_c membrane expression, IL-2 high-affinity binding and internalization, ligand binding-mediated phosphorylation of JAK3 and JAK1 tyrosine kinases, as well as IL-2–induced cell proliferation (Candotti et al., 1996; Hacein-Bey et al., 1996; Taylor et al., 1996). More importantly, normal development of mature T lymphocytes (Hacein-Bey et al., 1998) and NK cells (Cavazzana-Calvo et al., 1996) was demonstrated from gene-corrected X-SCID HSCs in vitro. In addition, in vivo experiments in mouse models of X-SCID have shown the safety and efficacy of γ_c gene transfer in bone marrow stem cells, with reconstitution of lymphoid development and function in these animals (Aviles Mendoza et al., 2001; Lo et al., 1999; Otsu et al., 2000, 2001; Soudais et al., 2000).

These results formed the basis for the first clinical gene transfer protocol for X-SCID, launched in 1999 in Paris, France. This trial involved retroviral γ_c gene transfer into $CD34^+$ bone marrow cells harvested from X-SCID patients who lacked HLA-identical bone marrow donors and enrolled 10 typical X-SCID patients. The results of this trial have been reported and showed that as soon as 60 to 90 days after the infusion of gene-corrected cells, T lymphocytes expressing γ_c appeared in the circulation and reached normal values within 4 to 8 months in eight patients (Cavazzana-Calvo et al., 1996; Ginn et al., 2005; Hacein-Bey-Abina et al., 2002, 2003a, 2010). Most importantly, these T lymphocytes were polyclonal and functionally competent, as demonstrated by normal responses to stimulation with mitogens and specific antigens. In contrast with the T lymphocytes, the NK cell counts declined to values lower than normal after an initial significant rise during which restoration of NK cell function was observed. In addition, despite the fact that virtually no B cells carried the γ_c transgene 6 to 10 years after gene therapy, close-to-normal serum levels of IgG, IgA, and IgM were demonstrated in four, five, and seven patients, respectively. All patients initially developed specific antibody responses to tetanus and diphtheria toxins, as well as poliovirus vaccines, but positive titers were not consistently detected in the long term, and three patients required immunoglobulin replacement therapy. Not surprisingly, based on the predicted lack of selective advantage in myeloid lineages, only 0.01 to 1 percent of the patients' monocytes and granulocytes showed evidence of genetic correction (Cavazzana-Calvo et al., 2001; Hacein-Bey-Abina et al., 2002, 2010; Sun et al., 1997).

This series of patients clearly demonstrated that γ_c gene transfer can restore the immune system in X-SCID patients. Although the restoration was incomplete with regard to the numbers of gene-corrected B and NK cells, the effects of the treatment were sufficient to provide protective immunity and to allow the patients to return home and lead a normal life. The failure of the treatment in 2 of the total of 10 patients treated in Paris was attributed to the specific characteristics of these two patients. The first subject had chronic infections that may

have affected the engraftment of gene-corrected cells (Hacein-Bey-Abina et al., 2002), while the second patient, treated in Australia, received a low number of gene-corrected cells and suffered from a significant viral infection early after treatment (Ginn et al., 2005). In addition, one of these two patients was an atypical patient of 15 years of age at the time of treatment, thus raising the possibility that loss of thymic function may have contributed to the failure of the procedure in this case.

Starting late in 2002, a series of four patients who had been treated with gene therapy about 3 to 5 years earlier presented with uncontrolled clonal T-cell proliferation. The malignant behavior of such proliferation required conventional treatment for T acute lymphoblastic leukemia to be administered, in one case followed by matched unrelated BMT.

In all cases, the adverse event was the result of insertional oncogenesis due to the aberrant expression of the *LMO2* (LIM domain only-2) or *CCND2* (cyclin D2) oncogenes induced by the integration of the γ_c retroviral vector in the proximity of the gene regulatory regions (Hacein-Bey-Abina et al., 2003a, 2003b, 2008). LMO2 is required for normal hematopoiesis and is activated in childhood acute T-cell leukemias by chromosomal translocation (Larson et al., 1994). CCND2 is normally active during early thymopoiesis and downregulated thereafter, which implicates its overexpression in T-cell leukemia development. Although our understanding of the mechanism underlying the development of LMO2- and CCND2-mediated lymphoproliferation remains incomplete, it appears plausible that the enhancer activity of the retroviral vector promoter was responsible for the activation of the LMO2 and CCND2 promoters and consequent deregulated LMO2 and CCND2 gene expression. Vigorous cell proliferation of LMO2- and CCND2-expressing cells due to the selective advantage conferred to them by the concomitant expression of the γ_c gene may have provided a "second hit" and favored secondary changes leading to the onset of the overt malignancies.

Whether or not the leukemic transformation observed in these patients can thus be attributed to a specific cooperation between γ_c and LMO2/CCND2 in the context of a strong selective advantage conferred by the genetic correction remains to be established, but it is obviously of critical importance as it could change the assessment of the risk that similar events may occur after retroviral-mediated gene transfer into HSCs for other diseases. These serious adverse events have had major repercussions on the field of gene therapy in general. It is clear that a complete reassessment of the risks of gene therapy for X-SCID and of the validity of the previous safety studies in animal models is warranted to provide an understanding of how to safely continue clinical experimentation of an approach that has provided clear clinical benefit but also unintended severe complications.

Despite aggressive therapy, one of the four patients who developed lymphoproliferation died, while the others remain in remission up to 10 years after the occurrence of the severe adverse event and conventional chemotherapy (Hacein-Bey-Abina et al., 2010).

In July 2001, a second gene therapy trial for X-SCID was started by Thrasher et al. at the Great Ormond Street Hospital in London, UK. The protocol design was similar to the French trial, with the exception of slight differences in culture conditions (lower concentration of IL-3 and serum-free medium) and the use of a gibbon-ape leukemia virus (GALV) pseudotyped vector. During the following 4.5 years, 10 children (4–46 months of age) were enrolled and treated; all patients had substantial improvements of clinical features and immunological function. The latter included recovery of T-cell numbers (up to normal numbers in six patients), normal T-cell proliferative responses (except in one patient), and a polyclonal T-cell receptor (TcR) Vβ repertoire (in six subjects). Improvement of humoral immunity was also observed that allowed discontinuation of prophylactic immunoglobulin replacement treatment in four patients (Gaspar et al., 2004, 2011b). Altogether, these data confirm the results obtained in the French trial and corroborate the notion that gene therapy can be curative for X-SCID. Also similar to the French trial, one patient developed LMO2-overexpressing T-cell leukemia about 24 months after treatment and responded to standard chemotherapy (Howe et al., 2008).

The French and British groups have also described the failure of their gene therapy protocol in two older X-SCID patients (20 and 15 years old) who had presented later in life with attenuated clinical phenotype. Neither patient showed significant changes of immunological parameters despite successful transduction of $CD34^+$ bone marrow cells and demonstration of transitory vector marking in B, NK, and myeloid cells in one patient. The authors hypothesized the compromised thymopoiesis in these older patients had posed restrictions to the efficacy of the procedure (Thrasher et al., 2005).

One additional trial was begun at the NIH in 2003 with the aim of offering a rescue treatment option for older X-SCID patients who had failed to respond to previous haploidentical BMT treatment. Three patients (11, 10, and 14 years old) were enrolled in this trial, which targeted G-CSF–mobilized peripheral blood $CD34^+$ cells. T-cell numbers and function significantly improved in the youngest subject, but no immunological improvement was observed in the other two subjects. Multilineage retroviral marking was documented in all three children (Chinen et al., 2007). The loss of thymic potential in these older patients may be a formidable challenge to the success of the procedure.

The occurrence of insertional oncogenesis in 20 percent of typical X-SCID patients treated with gene therapy vectors using the strong murine retroviral promoter/enhancer combination stimulated a series of effort to develop novel constructs to capitalize on the demonstrated efficacy of gene therapy for X-SCID while enhancing the safety of the procedure. Based on the notion that proto-oncogene activation was caused by enhancer-mediated promoter induction, an enhancer-less murine retroviral vector was generated and tested for efficacy in the mouse model of X-SCID (Thornhill et al., 2008). Based on the satisfactory results, a multicenter study including centers in London, Paris, Boston, Cincinnati, and Los Angeles has begun and had enrolled four patients as of December 2011. Data are available only for the first patient at about 6 months after treatment and are encouraging, showing increase of T-cell numbers to one third of normal values

and normalization of proliferative responses to mitogens (Pai et al., 2011). An alternative approach has been developed by Sorrentino and colleagues, who have elected to use a lentiviral vector to express the γc because of the reduced propensity of this class of vectors to integrate near gene transcriptional start sites (Wu et al., 2003). A two-site clinical trial using such vector (Zhou et al., 2010) is open at the St. Jude Children's Research Center in Memphis, where typical X-SCID patients will be enrolled, and at NIH, where atypical older patients will be treated. The latter arm of the trial will use nonmyeloablative conditioning to improve the efficacy of engraftment of gene-corrected cells.

JAK3 DEFICIENCY

The JAK3 member of the Janus family of protein tyrosine kinases is a lymphoid tissue-specific tyrosine kinase (Kawamura et al., 1994) involved in the signal transduction pathway of several cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Asao et al., 2001; Habib et al., 2002; Johnston et al., 1995). Patients with mutations of JAK3 usually present with autosomal-recessive SCID (Candotti et al., 1997; Macchi et al., 1995; Notarangelo et al., 2001; Russell et al., 1995) with clinical characteristics virtually identical to those presented by X-SCID patients, except for the fact that JAK3 deficiency can also be found in female patients (see Chapter 10).

Because of the clear similarities between the effects of γ_c and JAK3 genetic defects, the theoretical possibilities for gene therapy are analogous. Retrovirally mediated gene correction has reconstituted IL-2 and IL-4 responses in JAK3-deficient cell lines (Candotti et al., 1996; Oakes et al., 1996), leading to the hypothesis that the gene correction of HSCs may be a useful therapy for JAK3-deficient patients. Preclinical in vivo studies have demonstrated the feasibility of retroviral-mediated gene correction of JAK3 deficiency in murine models using irradiated adult recipients and nonirradiated newborn animals (Bunting et al., 1998, 1999, 2000). Of note, an in vivo selective advantage of transduced cells over untransduced JAK3-deficient counterparts was demonstrated for lymphoid lineages, suggesting that treatment procedures similar to those used in the gene therapy trial for X-SCID could lead to successful results in JAK3 deficiency. Based on these results, Sorrentino et al. at the St. Jude Children's Research Hospital in Memphis attempted the genetic correction of bone marrow $CD34^+$ cells of a JAK3-deficient patient who had failed to respond to BMT. Infusion of retrovirally transduced cells was attempted twice in this patient, resulting in low levels of marking without significant change of immunological status (Sorrentino et al., 2003). The reasons for unsatisfactory outcome are not clear at the moment, and it is hoped that further investigations in additional patients will clarify prospects for gene therapy for this disease.

WISKOTT-ALDRICH SYNDROME

WAS is characterized by eczema, thrombocytopenia, recurrent infections, autoimmune disorders, and a high incidence of lymphomas (Fischer, 1993; Sullivan et al., 1994). The breadth and severity of WAS symptoms make management of this disease a complex problem (Mullen et al., 1993). The WAS gene encodes a highly proline-rich protein, WASp (Derry et al., 1994), that has been implicated in actin organization in the cytoskeleton (see Chapter 43). The unsatisfactory results of haplodentical BMT in WAS patients (Antoine et al., 2003; Filipovich et al., 2001; Moratto et al., 2011; Ozsahin et al., 2008) justify efforts to test the safety and efficacy of gene therapy for this disease.

Studies in WAS lymphoblastoid B cell lines (Candotti et al., 1999; Huang et al., 2000), as well as T-cell lines and primary T lymphocytes (Dupre et al., 2002; Wada et al., 2002; Strom et al., 2003) have shown that retroviral-mediated gene transfer can correct the phenotype of WAS cells. More recent experiments have shown that *WAS* gene transfer using lentiviral vectors can provide functional correction of WAS patients' T cells with higher efficacy (Dupre et al., 2004). More importantly, in vivo models of gene therapy using knockout mice have shown that T-cell numbers and responses can be improved in animals treated with both retroviral and lentiviral *WAS* gene transfer, albeit at reduced levels compared to wild-type animals (Blundell et al., 2008; Bosticardo et al., 2011; Catucci et al., 2011; Charrier et al., 2004; Dupre et al., 2006; Klein et al., 2003; Marangoni et al., 2009; Strom et al., 2002). Interestingly, competitive repopulation experiments have also documented a selective advantage of wild-type over *WAS* knockout cells (Strom et al., 2002), thus suggesting that gene-corrected lymphocytes could similarly have a selective advantage over unmodified populations after gene therapy. These observations are in line with the reports of somatic reversion of *WAS* mutations in T-lymphocyte progenitors of WAS patients that resulted in significant numbers of WASp-expressing circulating T lymphocytes with selective survival advantage in vivo over WASp-negative cells (Wada et al., 2001, 2003, 2004).

Following up on in vitro functional restoration experiments of human hematopoietic progenitors using a MoMLV-based retroviral vector (Dewey et al., 2006), the first clinical trial of gene therapy for WAS started in Germany in 2006 and enrolled 10 patients in the following 3 years. Mobilized peripheral blood $CD34^+$ cells were transduced (with transduction efficiency about 50 percent) and reinfused into patients after conditioning with busulfan (8 mg/kg). The procedure resulted in a significant increase of WASp-expressing platelets and lymphocytes over time, up to 80 to 90 percent. Treated subjects showed a significant increase of platelet numbers and normalization of T-cell numbers and function, NK-cell function, immunoglobulin production, and responses to vaccines. One patient failed to respond to treatment because of an insufficient cell dose. All other patients markedly improved their clinical status, including bleeding tendency, susceptibility to infections, and autoimmunity. Unfortunately, insertional oncogenesis mediated by LMO-2 activation occurred in four patients and resulted in T-cell leukemia (Boztug et al., 2010; Braun et al., 2011).

More recently, a multicenter gene therapy trial using the lentiviral construct extensively tested in the mouse models described above opened in Milan, London, Paris, and Boston.

It is hoped that the increased theoretical safety of this class of vectors will protect patients from the occurrence of leukemic adverse events, and efficacy results are eagerly awaited by the scientific and clinical communities.

PRECLINICAL EXPERIMENTS OF GENE THERAPY FOR OTHER IMMUNODEFICIENCIES

PURINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCY

In most cases, the immunological disorder associated with genetic deficit of purine nucleoside phosphorylase (PNP—EC 2.4.2.1) is restricted to the T-cell compartment, while B-cell functions are generally normal (see Chapter 14). Enzyme replacement therapy, by means of irradiated red blood cell transfusions (Rich et al., 1980), has not been definitively proved to be beneficial for PNP deficiency, and allogeneic HLA-matched BMT is the current therapy of choice, although reports indicating its success in patients with PNP deficiency are limited.

The lack of PNP activity leads to the block of the same purine salvage pathway that is affected by the genetic deficit of ADA. The biochemical defects of ADA and PNP deficiency are similar, so the prospect for successful gene therapy of PNP deficiency also seems likely.

In preclinical studies using retroviral- and lentiviral-mediated gene transfer, in vitro reconstitution of PNP activity in T lymphocytes derived from PNP-deficient subjects has been described (Liao et al., 2008; Nelson et al., 1996). These results suggested that T cells could potentially be used as vehicles for therapeutic PNP gene expression, and clinical protocols testing this hypothesis have been proposed but not implemented. Contrary to ADA-SCID, no enzyme replacement treatment is available for this disease. PNP-deficient patients are usually lymphopenic; for this reason, the possibility remains that PNP-deficient patients will not have a sufficiently developed peripheral T-cell repertoire to achieve meaningful immune reconstitution with T-lymphocyte–directed gene therapy. Therefore, the correction of the gene defect at the level of HSC seems the best strategy, and the preliminary partial restoration of the PNP-deficient mouse model using bone marrow stem cell-directed gene correction (Liao et al., 2008) provides a platform for further development of the approach.

ZAP-70 DEFICIENCY

Mutations affecting the expression of ZAP-70 protein tyrosine kinase are a rare cause of autosomal recessive SCID characterized by absence of mature circulating $CD8^+$ T lymphocytes and TcR signal transduction defects in peripheral $CD4^+$ T cells (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994) (see Chapter 15). Since ZAP-70–deficient patients have been successfully treated by allogeneic BMT, this disease is another candidate for stem cell gene correction. The presence of circulating T lymphocytes in these patients has allowed the establishment of HTLV-1–transformed T-cell lines that have been used as targets for corrective gene transfer as a preliminary study of the feasibility of a gene therapy approach for this disease. Upon retroviral-mediated gene transduction of the normal ZAP-70 cDNA into T cells from a ZAP-deficient patient, it was possible to achieve expression of ZAP-70 kinase that was appropriately tyrosine phosphorylated upon TcR engagement. More importantly, reconstitution of in vitro kinase activity of the transduced ZAP-70 and normalization of calcium mobilization after TcR stimulation in gene-corrected cells was demonstrated (Taylor et al., 1996). In following experiments, Steinberg and coworkers transferred the ZAP-70 cDNA into primary T lymphocytes obtained from ZAP-70–deficient patients. Upon gene correction, several biological functions were restored in these T cells, including TcR signaling, Ras-mitogen-activated proten kinase (MAPK) activation, and IL-2 secretion. In addition, and importantly, selective growth advantage of gene-corrected cells was observed after in vitro culture of transduced cells (Steinberg et al., 2000), suggesting that the genetic correction of few HSCs and/or lymphoid progenitor cells may generate significant numbers of corrected T lymphocytes and restoration of T-cell immunodeficiency.

Because of the normally restricted expression of the ZAP-70 protein to specific lymphoid lineages, evaluation of potential deleterious effects of the ectopic expression of ZAP-70 in cell types normally not expressing this protein is a concern. However, gene correction of bone marrow cells of ZAP knockout mice using a retroviral vector expressing the human ZAP-70 gene resulted in the development of mature, polyclonal and functional T lymphocytes, while expression of the retroviral transgene in B cells did not affect their function (Otsu et al., 2002). Altogether, these data indicate that retroviral-mediated transfer of the ZAP-70 gene may prove to have a therapeutic benefit for patients with ZAP-70 deficiency.

To avoid some of the technical difficulties of HSC gene therapy, investigators have developed gene therapy approaches for ZAP-70 based on in situ gene correction of T-lymphoid progenitors in the thymus. This approach has been pursued using lentiviral vectors (Adjali et al., 2005) or simple electroporation of the correcting gene sequences (Irla et al., 2008). Intrathymic injection of a lentiviral vector or electroporation of ZAP-70 sequences resulted in the long-term differentiation of mature and functional thymocytes (Adjali et al., 2005; Irla et al., 2008), thus representing potentially safer alternatives to ex vivo gene-modified HSC gene therapy.

MHC CLASS II DEFICIENCY

Deficit of expression of MHC class II molecules can be caused by defects of several different MHC class II-specific transcription factors including CIITA, RFX5, RFXAP, and RFXANK. MHC class II deficiency is characterized by severe immunodeficiency with low numbers of $CD4^+$ lymphocytes (see Chapter 16). Allogeneic BMT is potentially curative, but its success rate for this disease is very low (Huss et al., 1995; Klein et al., 1995). Alternative forms of therapy are therefore needed, and HSC-directed gene therapy could be beneficial for these patients. Exploring this possibility, Bradley et al. have

shown that retrovirus-mediated gene transfer of the CIITA factor could restore expression of MHC class II in B lymphoblastoid lines and peripheral blood cells from an MHC class II-deficient patient (Bradley et al., 1997). Lentiviral vectors have also been described that express each of the above four MHC class II-specific transcription factors (Matheux et al., 2002); these may prove useful for future gene therapy applications. A cautionary note, however, comes from reports indicating that ectopic expression of MHC class II genes can result in pathological hyperactivity of $CD4^+$ lymphocytes (Bottazzo et al., 1986). Since expression of MHC class II molecules is regulated in important cell types such as T lymphocytes, fibroblasts, and endothelial cells, it will be important to determine whether tissue-specific expression of the therapeutic gene is needed for clinical applications.

IMMUNODEFICIENCY WITH HYPER-IGM

The X-linked hyper-IgM syndrome (X-HIM) is another rare PID caused by genetic mutations in the CD40 ligand molecule (CD40L) expressed on activated $CD4^+$ T lymphocytes (see Chapter 26). X-HIM patients lack functional CD40L on their T cells and have profound impairment of interactions between T cells and B cells. A detailed follow-up of X-HIM patients has made clear that the immunoglobulin substitution did not provide an adequate long-term solution to the severe infectious episodes suffered by these boys. Allogeneic BMT is therefore now indicated as definitive treatment for this disease, which is also a potential candidate for gene therapy.

Unfortunately, however, CD40L expression is highly regulated and restricted to activated $CD4^+$ lymphocytes, thus introducing the need for regulated gene transfer systems. The necessity of strictly regulating the expression of CD40L on gene-corrected cells was stressed by Brown et al., who performed transplantation of retrovirally gene-corrected bone marrow or thymic cells into CD40L knockout mice. Humoral and cellular immune responses of treated mice to influenza virus were partially or fully restored; however, more than 60 percent of these mice developed lymphoproliferative disease in the form of prelymphoma of T-lymphoblastic lymphoma. T-cell proliferation was most likely due to enhanced positive selection driven by the constitutive expression of CD40L on developing thymocytes (Brown et al., 1998). Gene transfer systems allowing effective regulation of CD40L therefore will have to be developed before gene-correction approaches can be proposed for the treatment of humans with X-HIM. One such approach has been proposed by Tahara et al., who aimed at preserving physiological regulation of CD40L by using spliceosome-mediated RNA trans-splicing. Bone marrow from CD40L knockout mice were transduced with a lentiviral vector encoding a pre-trans-splicing molecule containing wild-type CD40L exons 2–5 and transplanted into lethally irradiated recipient animals. Corrected CD40L mRNA, regulated CD40L expression in $CD4^+$ T cells, antigen-specific IgG1 responses, and attenuation of *Pneumocystis carinii* pneumonia were observed in treated mice in the absence of lymphoproliferative complications (Tahara et al., 2004). Although this strategy seems promising, further studies are needed to establish whether the efficacy of RNA trans-splicing will be adequate to correct the human phenotype.

Soluble CD40L is available and could represent an alternative form of therapy for X-HIGM. If proven beneficial, administration of autologous cells or "pseudo-organs" engineered to produce CD40L, but be easily removable, may become a more controllable option. As a more sophisticated approach, a lentiviral vector was recently constructed using the human CD40L proximal promoter to obtain tissue-specific, activation-inducible expression of CD40L. Testing experiments showed that transduced X-HIM T cells expressed CD40L only after stimulation with PMA+Ionomycin (Romero et al., 2011). These encouraging results suggest that CD40L-promoter-driven vectors may be able to be developed for gene therapy of X-HIM.

X-LINKED AGAMMAGLOBULINEMIA

Bruton's disease, X-linked agammaglobulinemia (XLA), is a genetic disorder of B-lymphocyte differentiation that results in absence of mature circulating B cells and undetectable serum immune globulins of all isotypes (Conley et al., 1994; Smith et al., 1994) (see Chapter 25). The molecular defect of XLA has been identified in mutations of the gene encoding Bruton's tyrosine kinase (*BTK*) (Tsukada et al., 1993; Vetrie et al., 1993), normally expressed by developing B and myeloid cells, but not plasma cells or T cells. The current management of XLA is with administration of immunoglobulin, which is generally efficacious in preventing infections. This treatment, however, is expensive, inconvenient, and in some cases insufficient to avoid lethal infections.

XLA patients therefore could benefit from development of gene therapy. The generation of retroviral vectors expressing *BTK* has been attempted since the mid-1990s, but several laboratories failed to produce high-titer retroviral-producing cell lines. These difficulties were explained by the demonstration that expression of *BTK* can mediate apoptosis (Islam et al., 2000). These findings suggest that high levels of *BTK* expression in target cells may induce toxic effects and that vector selection could be critical for the development of successful gene therapy for XLA. On the other hand, progress in vector design and production has made possible preclinical experiments of genetic correction in the X-linked immunodeficiency (xid) mouse, in which *btk* is mutated. Preliminary results showed that gene transfer into xid bone marrow cells can reconstitute B-cell development and production of IgM and IgG_3 antibodies (Conley et al., 2000; Yu et al., 2000). In addition, BTK-expressing B-cell progenitors were found to have a selective advantage over BTK-negative progenitors (Rohrer & Conley, 1999). Mice deficient in both Btk and the related kinase Tec have been corrected by retroviral-mediated gene therapy with a human *BTK* vector (Yu et al., 2004). Treated mice showed restoration of both primary and peripheral B-lineage development, correction of serum IgM and IgG_3 levels, and restoration of T-independent immunity and B-cell proliferative responses.

Following efforts focused on the generation of lentiviral vectors able to obtain B-cell–specific expression of BTK

and avoid potentially detrimental ectopic BTK expression in other lineages that has been shown to result in myeloerythroid proliferation (Kerns et al., 2010; Moreau et al., 2008; Ng et al., 2010). These experiments showed correction of B-cell development and antigen-specific antibody responses in *Btk* knockout mice; overall, the results support the continued development of gene therapy for XLA.

RAG-1 AND RAG-2 DEFICIENCY

Patients with mutations of the recombination-activating genes *RAG1* or *RAG2* suffer from a from of SCID characterized by the lack of both T and B lymphocytes. HSCT is currently the only curative treatment, but it is only partially successful in the absence of an HLA-identical familial donor. Exploring the potential of gene therapy for these diseases is therefore justified. Initial experiments used RAG-2–deficient mice and genetic correction of HSC with a MoMLV-based retroviral vector. Development of T cells and B cells was observed in treated mice, accompanied by a diverse T-cell repertoire, normal proliferative responses to mitogens and alloantigens, and humoral responses to antigenic challenge. A selective advantage for transduced lymphoid cells was demonstrated by comparative provirus quantification in RAG-2 lymphoid and myeloid cells, while there was no evidence of toxicity due to constitutive expression (Yates et al., 2002). The same investigators followed up with similar experiments in the RAG-1–deficient mouse model and showed that retroviral gene transfer into HSCs can restore normal B- and T-cell function, provided that high copy numbers of the transgene (≥5 copies/cell) were achieved in treated mice. These results suggested that a minimum threshold of RAG-1 expression may need to be achieved for efficient lymphoid development. In contrast to the RAG-2 model, however, high transgene copy number in lymphoid organs was associated with the occurrence of a lymphoproliferation event in one mouse, which raised the concern of a delicate balance between efficacy and toxicity for the application of retroviral-mediated gene correction of RAG-1 deficiency (Lagresle-Peyrou et al., 2008).

Additional experiments form the same group used lentiviral gene-corrected bone marrow $CD34^+$ cells obtained from several RAG-1–deficient patients and engrafted them into NOD-SCID mice in xenograft experiments aimed at testing the restoration of human B-cell differentiation following gene correction. Again, the results showed that restoration of B-cell differentiation occurred only after transplantation of samples transduced with the higher vector copy numbers (4 copies/cell) (Lagresle-Peyrou et al., 2008).

More recent experiments used a lentiviral vector designed to provide optimized expression of RAG-1. Treated mice showed normal T- and B-cell development, serum Ig levels, TCR-mediated cell proliferation, and cytokine production. Antigen-specific antibodies were produced after in vivo challenge with trinitrophenyl keyhole limpet haemocyanin TNP-KLH. These results were achieved with less than 1 vector copy/cell in lymphoid organs, and no insertional mutagenesis was observed (Pike-Overzet et al., 2011), which encourages further development of this vector for clinical applications.

CD3 DEFICIENCY

The TcR is constituted by a glycoprotein heterodimeric complex containing TcRα and TcRβ, or TcRγ and TcRδ chains that associate with the CD3 complex composed of CD3γ, CD3δ, CD3ε, and CD3ζ (or CD3η) polypeptides. Selective defects of the expression of CD3γ have been reported in humans, resulting in only partial disorders of cellular immunity (Arnaiz-Villena et al., 1992) (see Chapter 11). Because of the presence of circulating T lymphocytes in these patients, a T-lymphocyte–directed gene therapy approach could be developed. Retroviral-mediated expression of CD3γ in mutant Jurkat T cells resulted in normalization of IL-2 production after TCR/CD3 engagement and TCR/CD3 downmodulation in response to PMA (Sun et al., 1997). Similarly, CD3γ introduction into peripheral blood T lymphocytes from CD3γ-deficient patients corrected the TCR/CD3 expression and downregulation defects but unfortunately also induced constitutive IL-2 synthesis, causing death of nontransduced T lymphocytes. CD3γ gene transfer into postthymic lymphocytes therefore can become detrimental to the host, and regulated expression will be necessary if gene therapy is to be developed for this disease.

X-LINKED LYMPHOPROLIFERATIVE SYNDROME

Mutations or deletions in *SH2D1A/SAP* gene result in the X-linked lymphoproliferative disease (XLP), a combined immunodeficiency associated with severe or fatal outcome after EBV infection (see Chapter 44). The SH2D1A/SAP protein is primarily expressed in T and NK cells (Czar et al., 2001; Parolini et al., 2000; Wu et al., 2001). Among T lymphocytes, cytotoxic T cells (CTLs) play a crucial role in the immune response to EBV. CTLs from XLP patients are characterized by reduced IFN-γ production in response to EBV-mediated stimulation and decreased cytotoxic activity against autologous lymphocytoid cell lines. These defects were restored using retroviral-mediated *SAP* gene transfer, thus opening the way to approaches for corrective gene transfer into CTLs as a potential form of therapy for this disease (Sharifi et al., 2004).

However, a HSC-direct gene therapy approach would likely be more beneficial and can be tested in *SAP* knockout mice that are available and resemble the human condition (Wu et al., 2001; Czar et al., 2001; Yin et al., 2003). A proof-of-principle study used lentiviral-mediated gene transfer to introduce the human SAP cDNA into HSCs from *SAP* knockout mice. This study demonstrated that full correction of NK-cell cytotoxity and partial recovery of NKT-cell development and humoral responses can be obtained with such an approach (Rivat et al., 2013). The lentiviral vector used in this study provided constitutive expression of SAP, which may be problematic as cell types such as HSCs and B cells do not physiologically express SAP. Whether or not enforced expression of SAP was responsible for the incomplete immune recovery seen in treated mice is unclear. However, vectors with

physiologically regulated SAP gene expression are currently under development and will be able to answer this question in the near future.

ARTEMIS DEFICIENCY

Mutations in *Artemis*, a gene critical for VDJ recombination, give rise to a form of SCID originally described in Athabascan-speaking Native Americans. As in other SCIDs, patients present early in life with a typical severe immunodeficient phenotype (viral or *Pneumocystis* pneumonia, persistent viral diarrhea, and growth failure). As a characteristic of this disease, hematopoietic cells and fibroblasts from these patients exhibit increased cellular radiosensitivity (see Chapter 13). HSCT is curative, and preparative regimens including irradiation are usually avoided. The limited availability of ideal donors and the increased frequency of complicating events after the transplant (Neven et al., 2009) justify the development of gene therapy approaches as an alternative treatment for this disease. Preclinical experiments using *Artemis* knockout mouse models and Artemis-expressing lentiviral vectors have been performed by several groups and have demonstrated that genetic correction of HSCs can rescue T- and B-cell development and proliferation responses and serum immunoglobulin and specific antibody production (Benjelloun et al., 2008; Mostoslavsky et al., 2006). A note of caution was raised by studies that demonstrated toxic effects of Artemis overexpression on fibroblasts and 293 cells, including decreased cell viability, increased DNA damage, and increased apoptosis (Multhaup et al., 2010). These results point to a challenge in the clinical development of gene therapy for Artemis deficiency and suggest that future vector design should include endogenous transcriptional elements of the Artemis gene, which would achieve physiological regulation of the transgene in gene-corrected cells.

RETICULAR DYSGENESIS

Reticular dysgenesis, an autosomal recessive from of SCID caused by mutations of the adenylate kinase 2 (*AK2*) gene, accounts for 3 percent of all SCID cases (see Chapter 18). Characteristics of the disease include virtual absence of neutrophils, T lymphocytes, and NK cells, combined with variable decreases in red blood cell, platelet, and B-cell counts. In addition, affected newborns have bilateral sensorineural deafness. Allogeneic HSCT can correct hematopoiesis, but myeloablative conditioning is required to achieve engraftment of myeloid lineages. As for other forms of SCID, treatment options using autologous gene-corrected HSCs would be desirable. Preliminary work toward gene therapy for reticular dysgenesis involved lentiviral vector constructs and transduction of patient-derived bone marrow cells. These experiments showed that AK2 gene transfer can overcome the promyelocyte/myelocyte differentiation arrest in vitro and allow the production of mature neutrophils (Lagresle-Peyrou et al., 2009). Additional experiments will be necessary to verify that, in addition to granulopoiesis, correction of AK2 expression can also rescue lymphocyte development. Unfortunately, these experiments are hampered by the current absence of a mouse model of the disease.

CONCLUSIONS

PIDs fully meet the definition of "experiments of nature" as proposed by Garrod (1924): they provide unique examples of the real consequences of genetic defects in humans, thus shedding light on the physiological role of the missing gene product(s). For this important characteristic, the highly heterogeneous group of extremely rare PIDs has been the focus of extensive clinical and molecular studies and has often been the object of innovative therapeutic approaches.

The first demonstration that allogeneic HSCT could be successfully applied to clinical practice was reported in 1968 following the treatment of a SCID child and a WAS patient (Bach et al., 1968; Gatti et al., 1968). Similarly, successful enzyme replacement therapy was first achieved in a case of SCID (Pahwa et al., 1989). Finally, SCID was also the first disease to be successfully treated by gene therapy (Cavazzana-Calvo et al., 2000). It is not surprising, therefore, that other cutting-edge technologies have continued to be bench-tested on PID models, such as the early demonstrations of the *IL2RG* gene repair with zinc-finger nuclease technology (Urnov et al., 2005) and the more recent development of site-directed gene-addition strategies for CGD in induced pluripotent stem cells (iPSc) (Zou et al., 2011).

With the first clear clinical benefits, the first serious potential complications of gene therapy have unfortunately also become evident (Hacein-Bey-Abina et al., 2003a). This has sparked a revision of the assessment of risks and benefits that will be critical for future development of gene therapy approaches for all PIDs and other genetic disorders.

As HSCT has subsequently emerged as an essential form of treatment for a wide variety of clinical applications, it is likely that the experience acquired from the pioneering gene therapy experiments for immunodeficient patients will constitute a guide for developing and applying gene therapy strategies to an increasing number of congenital and acquired genetic conditions in which conventional therapies have failed or are not a satisfactory long-term option.

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