Manzoor M. Khan

Immunopharmacology

Second Edition

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Preface

Since the publication of the first edition, there have been significant advances in the understanding of the basic function of the immune response and their relevant clinical applications. Some of the key discoveries include the identification of the new subset of helper T cells, new cytokines and their networks, and novel signal transduction mechanisms. For example, the identification of TH17 subset of helper T cells, in addition to TH1 and TH2 cells, not only advanced our understanding of the function of the basic immune response but our awareness of the possible etiology and pathogenesis of diseases such as allergy, asthma, rheumatoid arthritis, and other autoimmune/immune system-based diseases. The newly identified powerful cytokine networks that regulate both innate and acquired immune responses emerged as a result of the finding of the new cell types such as innate lymphoid cells and iNKT. Identification of the new cytokines and their networks has advanced our knowledge of the mechanisms involved in the maintenance of tissue homeostasis, including inflammation and tissue repair during stress and injury.

From the clinical application's perspective, there have been significant advances in oral immunotherapy for allergic disease, the treatment of HIV infection, the development of new monoclonal antibodies and their fragments to treat human diseases, and the immune cell-based therapies for cancer. The development of HIV vaccines has seen dramatic changes over the last few years. There has been a shift from a sole focus on T-cell vaccines to a holistic approach that pertains to the induction of both humoral and cellular elements. This entails induction of antibodies – both binding and neutralizing – to prevent infection. The cellular vaccination produces a safety net of CD8 T-cell responses to suppress the replication of the virus in the infected patients; as both of the effector arms are aided by helper T cells. The concept of immunotherapy was in infancy when the first edition was written, and since then major advances have been made not only with several major clinical trials but also with the approval of Sipuleucel-T by the FDA for the treatment of cancer in 2010. Furthermore, CAR T-cell therapy to treat cancer is in infancy with great expectations. As a result, the gap between early scientific knowledge and the late development of immune-based therapies is gradually narrowing.

Consequently, the significance and magnitude of these advances warranted a revision of this contribution. The revised edition will provide an in depth understanding of the recent advances and discoveries of the function on the immune response and their applications in the development of novel therapies to treat human diseases.

As we entered the twenty-first century, major advances in the arena of recombinant DNA, hybridoma, and transgenic technologies had revolutionized not only the understanding of the etiology and pathogenesis of a number of debilitating and life-threatening diseases but also provided novel modes of treatment. Whether it is the clinical application of recombinant cytokines, their agonists or antagonists, monoclonal antibodies, regulatory T cells, gene therapy, or the concept of T-cell vaccines, these all required the understanding of an evolving discipline that worked on the interface of immunology, pathology, pharmacology, and genetics called immunopharmacology. The initial emphasis of the discipline was the development of the drugs, which suppressed immune response to prevent tissue rejection after organ transplantation. The field, once considered restricted only to protect the host from invading organisms by mounting immune and inflammatory responses, evolved exponentially as we gradually learned about the exciting and sometimes adverse role of the products of the immune response in a very wide range of physiologic and pathologic settings ranging from cardiovascular, pulmonary, and gastrointestinal to neurological functions. A number of these products and therapies, based on their understanding continue not only to become symptomatic and curative therapeutic agents but have extensively contributed to the early diagnosis of a number of dreadful disorders.

This book is written for the graduate students in pharmacology and the professional students in pharmacy and medicine. The introductory chapter is aimed for the students who have not previously taken a course in basic immunology. Chapters 2 and 3 focus on cytokines, their receptors, pharmacology, and clinical applications. The next section is devoted to the pharmacology of immune regulatory agents, monoclonal antibodies, etiology, mechanisms of IgE-mediated responses, and immunotherapy for allergic disease. The following section includes chapters on the mechanisms of allograft rejections with description of the requirements for different types of clinical tissue transplantation and immunologic basis of acquired immunodeficiency disease. In the chapter on AIDS, the emphasis has been on the life cycle of HIV, available therapeutic options and the difficulties associated with the development of a vaccine for AIDS, and why an HIV vaccine does not fit the paradigm for the classical vaccine development. The last part of the book includes chapters on regulatory T cells and their therapeutic potential followed by the last chapter on the challenges and use of gene therapy to treat human disease.

Omaha, NE, USA Manzoor M. Khan
April 1, 2016

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Chapter 1
Overview of the Immune Response

Abstract This chapter introduces the components of the immune response that includes differences in innate and acquired immune systems and their differences. Specifically, physical, chemical, and cellular barriers, complement system, and Toll-like receptors are discussed in reference to the innate immune system, whereas humoral and cell-mediated responses are described when explaining the acquired/adoptive immune response. The details include the different isotypes of antibodies involved in humoral immune response and various types of cells that participate in humoral versus cell-mediated immune responses. Additionally, the concepts of antigen recognition, antigen presentation, and molecules involved in these processes, including the major histocompatibility complex, are described. Lastly, mechanisms of cellular migration and immune tolerance are discussed. The emphasis is that how the immune system recognizes and responds to the invading pathogens and what type of system is in place and sequentially develops to perform these tasks.

Keywords Immunogen • Antigen • Innate immunity • Physical barriers • Chemical barriers • Cellular barriers • Complement system • Toll-like receptors • Acquired immune response • Bone marrow • Thymus • Lymphoid organs • Antibodies • IgG • IgA • IgM • IgD • IgE • Immunoglobulins • Lymphoid cells • B cells • T cells • Helper T cells • Cytolytic T cells • TH1 • TH2 • TH9 • TH0 • TH17 • NKT cells • ϒŏ T cells • Memory T cells • Regulatory T cells • Natural killer cells • $CD4^+$ • $CD8^+$ • $CD3^+$ antigen-presenting cells • Macrophages • Dendritic cells • Neutrophils • Basophils • Eosinophils • Mast cells • Adhesion molecules • Integrins • Selectins • Cadherins • TCR • γδ TCR • TCR-1 • TCR-2 • Antigen recognition • Antigen processing • Antigen presentation • CD28 • CD40 • B7 • MHC molecules • HLA-A • HLA-B • HLA-C • HLA-D • HLA-DP • HLA-DR • HLA-DQ • MHC class I molecules • MHC class II molecules • Immune tolerance • Central tolerance • Peripheral tolerance

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1.1 Introduction

The immune system is a defense system that protects from infectious organisms and cancer. It is made up of a variety of cells, proteins, tissues, and organs. The cells participating in immune response are designed to recognize and eliminate invading agents. If not eliminated, these invading microorganisms may cause disease. The recognition is a very specific process that enables the body to recognize nonself molecules so the second phase of the process, an immune response, may initiate. Under normal circumstances, an immune response is not generated against self, which are the body's own proteins and tissues. The immune system for each individual is unique, and it employs small and efficient tools to recognize invading organisms that lack a central control. However, they are widely distributed in the body. The recognition of the nonself is not perfect and absolute detection of every pathogen is not required. Immune system is able to recognize molecules, which it has never seen before and can produce an effective response against them. It has been suggested that this system is scalable, resilient to subversion, robust, and very flexible and degrades.

After the recognition of the nonself, the effector phase is generated, which is characterized by the generation of a response against the invading microorganism in which a variety of cells and molecules participate, resulting in the neutralization and/or elimination of the pathogen. Some memory is retained of that pathogen and a second exposure to the same organism results in the development of a memory response. This response has a quick onset and is fiercer, resulting in a more efficient elimination of the pathogen.

1.2 Immunogens and Antigens

Immunogens are any agent capable of inducing an immune response. Their characteristics include foreignness, high molecular weight, and chemical complexity. Antigens are any agent capable of binding specifically to the components of immune response. They include carbohydrates, lipids, nucleic acids, and proteins. Most immunogenic molecules, which induce an immune response, require both T and B lymphocytes. Because T lymphocytes mature in the thymus, these immunogens are called thymus-dependent antigens. Certain types of molecules can induce the production of antibodies without the apparent participation of T lymphocytes. These molecules are referred to as the thymus-independent antigens. The portion of an antigen that binds specifically with the binding site of an antibody or a receptor on lymphocytes is termed an epitope.

1.3 Components

1.3.1 Innate Immunity

This is a general protection that is also termed as natural immunity, which is present at birth in all individuals. A species is armed with innate immunity that provides an individual the basic resistance to disease. This is also called nonspecific immunity

and is the initial defense against infections. It is characterized as broad-spectrum responses, with limited repertoire of recognition molecules and a lack of memory component. Since it is the first line of defense, it is present at birth, it is nonspecific, and it does not allow an increase in resistance after repeated infections. It destroys vast amounts of microorganisms in a short time, with which an individual comes in contact with every day, and protects from causing the disease. There are three types of barriers for the innate immunity, physical barriers, cellular barriers, and chemical barriers.

1.3.1.1 Physical Barriers

The physical barriers include the skin, mucous membranes, epidermis, and dermis. The skin maintains a low pH because of lactic and fatty acids. The mucous membranes in the respiratory system, urogenital system, and gastrointestinal system create a substantial surface barrier. Epidermis and dermis constitute additional physical barriers. The dermis also produces sebum, which maintains an acidic pH due to its lactic and fatty acid content.

1.3.1.2 Chemical Barriers

A number of endogenous chemicals provide effective barriers in innate immunity. They include hydrolytic enzymes of saliva, low pH of the stomach and vagina, and proteolytic enzymes in the small intestine. Additional examples include cryptidins, α-defensins, α-defensins and interferons, and surfactant proteins A and D. Cryptidins and α-defensins are produced in the small intestine, and α-defensins are produced by the skin and respiratory tract.

1.3.1.3 Cellular Barriers

The cellular barriers include macrophages, dendritic cells, eosinophils, phagocytes, and natural killer cells. Some of these cells internalize macromolecules that they encounter in the circulation or in tissues. This internalization takes place either by pinocytosis, receptor-mediated endocytosis, or phagocytosis. The pinocytosis involves nonspecific membrane invagination. In contrast, receptor-mediated endocytosis involves specific macromolecules, which are internalized after they bind to respective cell surface receptors. Endocytosis is not cell specific and carried out probably by all cells.

As opposed to endocytosis, phagocytosis is more cell specific and results in the ingestion of particulate as well as whole microorganisms. The cells involved in phagocytosis include monocytes and macrophages, neutrophils, and dendritic cells. Furthermore, fibroblasts and epithelial cells can also be induced to assume phagocytic activity.

1.3.1.4 Complement System

The complement system is a part of the innate immune response that facilitates the ability of antibodies and phagocytes to clear invading organisms. Its ability remains the same and does not improve overtime. Nonetheless, it can play a supportive role in the acquired immune response. The complement system is composed of a number of small proteins present in blood. It is synthesized by the liver and circulates as pro-proteins, which are its inactive precursors. After induction, proteases in the complement system cleave specific proteins. This causes the secretion of cytokines and begins a series reactions culminating in enhanced response, as well as induction of the membrane attack complex, which is cytotoxic in nature. The complement system is composed of more than 35 proteins and protein fragments. They include serum, serosal, and cell membrane receptor proteins.

As shown in Fig. 1.1, the complement system is activated by three distinct biochemical pathways. These pathways include the classical complement pathway, the alternative complement pathway, and the lectin pathway. The hepatocytes, macrophages, monocytes, and epithelial cells of the gastrointestinal and urogenital tract synthesize the components of the complement system. The homologous variant protease C3-convertase is produced by all three pathways of activation. Antigen-bound antibody molecules drive the activity of the classical complement pathway. Six units of IgG with the exception of IgG4 or one unit of IgM can activate the complement pathway. The pathway is initiated after the Fc region of the antibody binds to C1 component. It can also be triggered by additional mechanisms such as the binding of the C-reactive protein to polysaccharides of microbial agents. C1 is the initial enzyme, which is made up of two subunits, with calcium-dependent interaction.

Antigens alone and C3 hydrolysis are the stimuli of the alternative complement pathway and the lectin pathway. The alternative pathway is in a continuous activated state, though at low levels. It does not depend on the binding of antibodies to the pathogen. Lectin pathway is homologous to the classical pathway and is activated by attachment of the mannose-binding lectin to mannose residues on the surface of the invading pathogen. This activates mannose-binding lectin-associated serine proteases. Component C3 is cleaved and activated by C3-convertase in all three pathways. This results in the formation of C3a and C3b, which catalyze a series of additional pathways. The internalization and opsonization of an invading organism by phagocytes is facilitated by binding of C3b to the surface of the pathogen. The recruitment of inflammatory cells is mediated by C5a that is an important chemotactic protein. Both C3a and C5a can degranulate mast cells and are anaphylactic in nature. They also cause contraction of the smooth muscles and increase vascular permeability. The formation of membrane attack complex involves the activation of membrane attack pathway orchestrated by C5b and includes C5b, C6, C7, C8, and C9. The membrane attack complex is cytolytic in nature and is the final product of the complement pathways. The osmotic lysis of the target cell is the result of the formation of a transmembrane channel by the membrane attack complex. Complement-coated invading organisms or their fragments are removed by the phagocytic cells.

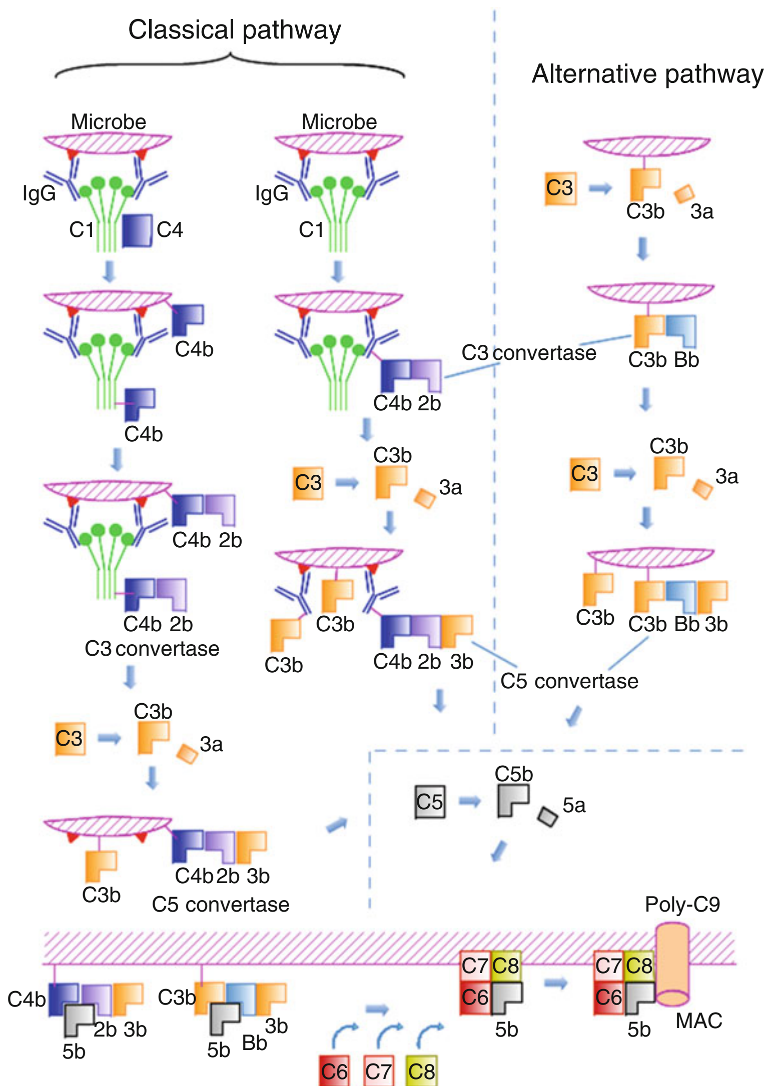

Fig. 1.1 The classical and alternative complement pathways with the late steps of complement activation (Source: Tossh_eng, under the terms of GNU free documentation license)

The functions of the complement system include opsonization (preparation to eat, increasing the ability of the phagocytes to remove the antigen), chemotaxis (attracting mono- and polymorphonuclear phagocytes), agglutination (clumping of the antigens), and cell lysis. Complement system could be deleterious to the host and thus needs to be strictly regulated. This is achieved by complement control proteins, which are present in blood plasma and host cells.

The role of complement system is suggested in a variety of immune-mediated diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune heart disease, inflammatory bowel disease, and Barraquer–Simons syndrome. Furthermore, there is evidence for the involvement of complement system in neurodegenerative conditions and other diseases of the central nervous system, including Alzheimer's disease. In HIV/AIDS the complement system is activated to cause additional damage to the self. Mutations and polymorphisms in complement system are responsible for a number of other disease states.

1.3.1.5 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of polypeptides, which are crucial in innate immunity. They are generally expressed on antigen-presenting cells, including macrophages and dendritic cells. TLRs recognize microbes and activate immune response. Their subtypes include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13. They are a type of pattern recognition receptors and recognize molecules, which pathogens share (pathogen-associated molecular patterns). In combination with a cytokine receptor (IL-1R), they form a receptor superfamily that is called "interleukin-1 receptor/Toll-like receptor superfamily." A common domain is shared by all members of this family, which is referred to as Toll-IL-1 receptor (TIR) domain. There are three subgroups of TIR domains. The receptors for cytokines that are produced by antigen-presenting cells (macrophages, monocytes, dendritic cells) form the first subgroup. Classical TLRs that bind to microbes form the second group. TIR domains consisting of cytosolic adopter proteins make up the third subgroup and are also involved in signaling of the proteins of the other two subgroups (Fig. 1.2).

The ligands for TLR include bacterial lipopolysaccharides (LPS), lipoproteins, flagellin, the unmethylated CpG islands of bacterial and viral DNA, double-stranded RNA of viruses, as well as other molecules. It is considered that TLRs act as dimers. For full ligand sensitivity, they may also rely on other co-receptors. TLR signaling is divided into two pathways, the MyD88-dependent and TRIF-dependent pathway. The MyD88-dependent response occurs on dimerization of TLR receptor and, with the exception of TLR3, is used by all other TLRs. It activates NF-κB and mitogen-activated protein kinase (MAPK). The adopter protein MyD88 is recruited after ligand binding and conformational changes in the receptor. IRAK1, IRAK2, and IRAK4 are then recruited by MyD88. TRAF6 is then phosphorylated and activated by IRAK kinases. The binding to IKK-β results from the polyubiquitination of TAK1 and IRAK kinases. After binding, TAK1 phosphorylates IKK-β, resulting in the phosphorylation and degradation of IκB, which allows migration of NF-κB into the cell nucleus. This cascade results in the transcription and translation of pro-inflammatory cytokines.

TRIF-dependent pathway is used by TLR3 and TLR4. dsRNA activates TLR3 pathway and LPS activates TLR4 pathway. The adaptor TRIF is recruited after the activation of TLR3 by dsRNA. TBK1 and RIPK1 are activated by TRIF. IRF3 is

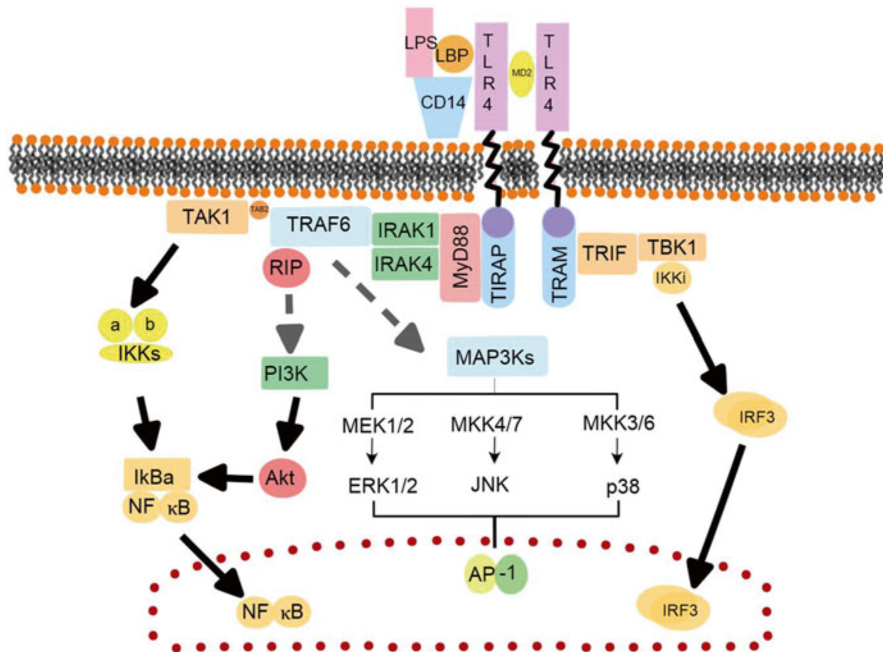

Fig. 1.2 Toll-like receptor pathways (Source: Niels Olson – Licensed under CC BY-SA 3.0 via Wikimedia Commons)

phosphorylated by the TRIF/TBK1 signaling complex. This results in the migration of IRF3 into the nucleus, where it causes the transcription and translation, eventually resulting in the production of type I interferon. TLR signaling results in the augmentation or inhibition of genes, which produce the inflammatory response. Signaling by TLR activates or regulates a very large number of genes. All four adaptors are used by TLR4 that is unique for this subset of receptors. TIR domain-containing adaptors TIRAP and MyD88 are recruited by TLR4, MD2, and LPS. This causes the activation of NF-κB and MAPK. A signaling complex is then formed between TRAM, TRIF adaptors, and TLR4-MD2-LPS complex, after the TLR4-MD2-LPS complex goes through endocytosis. IRF3 is activated as a result of this TRIF-dependent pathway. This results in the production of type I interferon. However, the production of pro-inflammatory cytokines requires both early and late phases of NF-κB activation. The signal transduction pathways for TLR are shown in Fig. 1.2.

There are multiple outcomes after TLRs are activated by microbes. In response to viral infection, the infected cell may undergo apoptosis. There may also be a release of antiviral cytokines, such as interferons. While TLRs are involved in the release of cytokines, they play no significant role in the phagocytosis of the microbes. In case of bacterial antigens, pro-inflammatory cytokines are produced. The invading pathogens may also be uptaken by the antigen-presenting cells and after undergoing processing presented to naïve helper T cells.

In addition to their role in innate immunity, TLRs also play a key role in acquired immune responses. Their functions are broad and they affect tissue homeostasis. TLR signaling has been implicated in many inflammatory diseases, including asthma, allergic rhinitis, autoimmune diseases, and other immune-mediated inflammatory diseases. The pathological manifestation may result from either overactive TLR signaling or insufficient signaling. A number of TLR ligands, both agonists and antagonists, are being studied in animal models, preclinical and clinical settings. Since TLRs work together, their function is complex. Our information for their therapeutic implications is still rudimentary and requires significant additional understanding.

1.3.2 Adaptive/Acquired Immune Response

This immune response occurs when the body encounters an antigen and/or pathogen. With this response, the body protects itself from future encounters with the same antigen/pathogen so they will not cause disease. The response is more complex than the innate immune response. It requires the recognition and processing of the immune response. After the antigen is recognized, the adaptive immune response employs humoral and cellular responses specifically designed to eliminate the antigen. This response also includes a memory component, which allows improved resistance against that specific antigen during subsequent infections. T lymphocytes, B lymphocytes, and macrophages participate in the acquired immune response. The lymphocytes (T and B cells) are central to all acquired immune responses, because of their specificity in recognizing the pathogens. This recognition can take place either inside the tissue or in blood or tissue fluids. B cells recognize antigens by synthesizing and releasing antibodies, which specifically recognize antigens. T lymphocytes do not secrete antibodies but have a wide range of regulatory and effector functions.

1.3.2.1 Antibodies

The antibodies are proteins that are produced during the immune response. They identify and eliminate foreign objects such as bacteria and viruses. They are synthesized in response to specific antigens and only bind to the antigen against which they are produced. Antibodies are glycoproteins and are also called immunoglobulins. They are synthesized and secreted by B lymphocytes, which produce antibodies after their activation resulting from exposure to an antigen. The antibodies can circulate freely or in the bound form attached to the cells, which possess Fc receptors.

The antibody molecule (monomer) is a Y-shaped structure (Fig. 1.3). It consists of two identical light and heavy chains, which are connected by disulfide bonds. In the native state, the chains are coiled into domains, each of which consists of 110

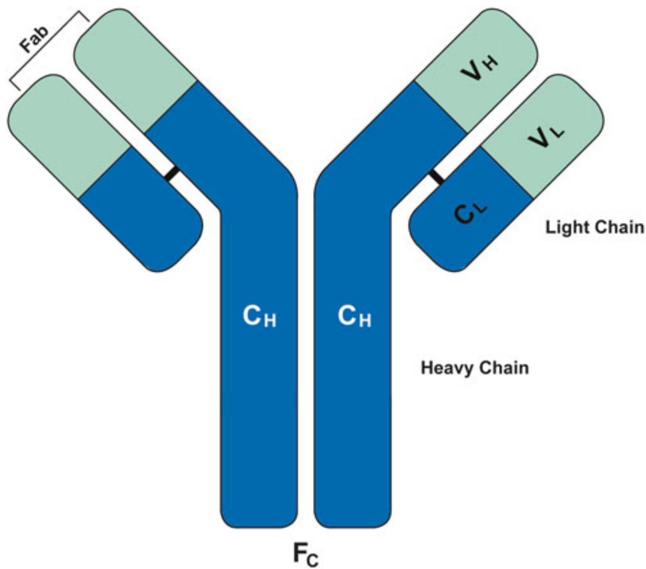

Fig. 1.3 The immunoglobulin molecule. Each immunoglobulin molecule is composed of two identical heavy (CH + VH) and two identical light chains (CL + VL). The antigen binds to the Fab region which varies according to the specificity of the antibody. The rest of the domains (*blue*) are constant. The classes of the antibody molecules differ based on the Fc region of the heavy chain

amino acids. Both light and heavy chains are made up of constant and variable regions. The two identical light (L) chains and two identical heavy (H) chains are held together by disulfide bonds. Both light and heavy chains have constant and variable regions. Two major classes of L chains are kappa and lambda and the ratio of κ and λ chains varies from species to species. Papain splits the immunoglobulin molecules into three fragments of about equal size. Two fragments are antigen-binding fragment (Fab) and the third fragment is fragment crystallizable (Fc). The variable regions of both heavy and light chains form the antigen-binding site (Fab). The Fab region varies according to the specificity of the antibody. The antibodies are very diverse molecules and their differences reside predominantly in the variable region. This variability enables each antibody to recognize a particular antigen.

There are five different types of heavy chains, which correspond with five different classes of antibodies. The constant region of the heavy chain is identical in all antibodies of the same class. The classes of the immunoglobulin molecules differ based on the Fc region of the heavy chain, which are responsible for the different functions performed by each class. Thus the constant region confers on each class of antibody its effector function.

As depicted in Table 1.1, there are five different classes of H chains. Each chain differs in antigenic reactivity, carbohydrate content, and biological function. The nature of the H chain confers the molecule its unique biologic properties. A distinctive set of glycoforms characterizes each immunoglobulin. The glycoforms render

Table 1.1 Classes (isotypes) of H chains

Immunoglobulin class (isotype)	Heavy chain
IgM	μ
IgG	γ
IgA	α
IgD	δ
IgE	ϵ

broad differences in the frequency, form, and locality of oligosaccharides, which are responsible for the diversity of immunoglobulins. Since these glycoform populations can be identified on a regular basis, any alteration in their characteristics suggests a disease state and could be a potential therapeutic tool. The oligosaccharides possess critical recognition epitopes. This provides the immunoglobulins with additional functional repertoire. The effector function of immunoglobulins is thus regulated by these sugar molecules.

The antibody molecules have two distinct functions. The first is to bind the pathogen such as virus or the bacteria against which immunoglobulin was produced, and the second is to recruit other cells and molecules, such as phagocytes or neutrophils, to destroy the pathogen to which the antibody is bound.

1.3.2.2 Classes of Immunoglobulins

There are five different classes of immunoglobulins: IgG, IgM, IgA, IgD, and IgE. The structures are shown in Fig. 1.4.

IgG: Immunoglobulin G is present in lymph fluid, blood, cerebrospinal fluid, and peritoneal fluid. It is composed of 2 γ chains of 50 kD and 2L chains (κ or λ) of 25 kD with a total molecular weight of 150 kD. The functions of IgG include agglutination and formation of precipitate, passage through the placenta and thus conferring immunity to the fetus, opsonization, antibody-dependent cell-mediated cytotoxicity (ADCC), activation of complement, neutralization of toxins, immobilization of bacteria, and neutralization of virus.

IgM: The gene segment that encodes the μ constant region of the heavy chain occupies the front position among other constant region gene segments, and consequently, IgM is the first immunoglobulin produced by mature B cells. Its molecular weight is 900 kD and is a five-chain structure. All chains consist of 2L and 2H chains and have five antigen-binding sites. It is synthesized in appreciable amounts by children and adults after immunization or exposure to thymus-independent antigens. Elevated levels usually indicate a recent infection. IgM does not pass across the placenta but is synthesized by the placenta. Its elevated levels in the fetus are indicative of congenital infection. It is the best agglutinating and complement-activating antibody and possesses high avidity. Sometimes it is referred to as a natural antibody, since it is often bound to specific antigens, even when there was no prior immunization.

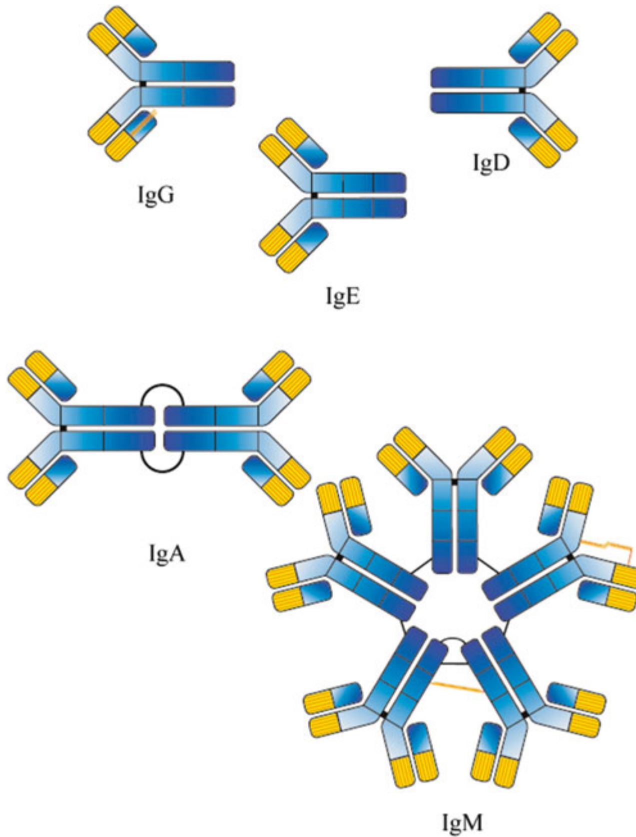

Fig. 1.4 Classes of immunoglobulins. This figure depicts five classes of immunoglobulins IgG, IgE, IgD, IgA, and IgM. IgA can be present as a monomer or dimer molecule, whereas IgM exists as a pentamer

IgA: Immunoglobulin A is a major immunoglobulin in external secretions (saliva, mucus, sweat, gastric fluid, and tears). It is a major immunoglobulin of colostrum and milk; it has a molecular weight of 165 kD and is present in both monomeric and dimeric form. It is present in two isotypes, IgA1 (90 %) and IgA2 (10 %). The bone marrow B cells produce IgA1, which is present in serum. The B cells located in the mucosa synthesize IgA2, which is present in secretions. Chemically the heavy and light chains of IgA2 are bound by non-covalent bonds and not connected by disulfide bridges. It plays an important role in mucosal infections, bactericidal activity, and antiviral activity. Plasma cells produce polymeric IgA, and mucosal epithelial cells express polymeric Ig receptor, resulting in high levels of IgA in mucosal areas. This is followed by its transportation across mucosal epithelial cells, where it is separated from its receptors resulting in its release into secretions. Its effects are achieved after interaction with specific receptors including FcR1, Fcμ/micro R, and CD71. However, certain pathogens block the protective properties of IgA.

IgD: Immunoglobulin D causes the differentiation of B cells to a more mature form and is expressed on the surface of B lymphocytes. It is present in a monomeric form with a molecular weight of 180 kD.

IgE: Immunoglobulin E is associated with type 1 hypersensitivity and allergic disease. Its molecular weight is 200 kD. It also plays a role in host defense against parasitic infections. IgE binds to specific Fc receptors on the cell surface of mast cells, basophils, eosinophils, macrophages, monocytes, and platelets. Two main types of Fc receptors for IgE include FcεRI and FcεRII. The former is a high-affinity receptor, whereas the later, also termed as CD23, is a low-affinity receptor. FcεRI receptors are present on mast cells and basophils, whereas FcεRII are present on B cells, although their expression can also be induced on other cell types including monocytes, macrophages, eosinophils, and platelets by TH2 cytokine, interleukin (IL)-4. IgE serves as a stimulus for the upregulation of both receptors. Binding of IgE to its receptors on mast cells results in the release of a variety of endogenous mediators including several cytokines, and the symptoms can vary from a mild allergic response to potentially life-threatening anaphylactic shock. Normal physiologic levels of IgE are low, but under atopic conditions, its levels rise as a result of an isotype switch from IgG to IgE. This is in response to an antigen and under the influence of TH2 cell-derived cytokines.

1.4 Cell Cooperation in the Antibody Response

After exposure to an antigen, its recognition by the immune system is followed either by production of an immune response or the development of tolerance, depending on the circumstances. The immune response could be humoral, cell mediated, or both. On second and subsequent encounters with the same antigen, the type of response is determined by the outcome of the first response. However, the quantity and quality of both responses are very different.

1.4.1 Primary and Secondary Antibody Responses

After administration of an antigen for the first time, there is an initial lag phase where antibodies are not produced. This is followed by a period in which the antibody titer rises logarithmically to a maximum and subsequently declines. The decline is due to either the breakdown or clearance of the antibodies.

The primary and secondary responses differ in four ways:

1. *Time course*. The secondary response has a shorter lag phase and an extended plateau and decline.
2. *Antibody levels*. The antibody levels are ten times higher in the secondary response as compared to the primary response.

3. *Antibody class*. The major proportion of the primary response is made up of IgM, whereas the secondary response consists almost entirely of IgG.
4. *Antibody affinity*. The affinity of the antibodies is much greater in the secondary response as opposed to the primary response, which is termed as "affinity maturation."

1.5 Cells Involved in the Immune Response

The immune system is composed of a variety of different cell types and organs that are involved in specifically recognizing nonself antigens to eliminate them. Phagocytes are an important defense, which participates in both innate and acquired immune responses. The lymphoid cells render the high degree of specificity involved in the recognition of nonself antigens and are part of the acquired immune response. All cells participating in the immune response arise from pluripotent stem cells and are divided into the lymphoid lineage – consisting of lymphocytes – and the myeloid cells, consisting of phagocytes (monocytes and neutrophils) and other cells.

There are three different kinds of lymphocytes that have specific functions: T cells, B cells, and natural killer cells. T cells develop in the thymus, while B cells develop in the adult bone marrow. The thymus and the bone marrow are the primary lymphoid organs where lymphocytes acquire specific cell surface receptors, which give them the ability to recognize antigens. The natural killer cells are cytotoxic lymphocytes that develop in the bone marrow. The phagocytes are made up of either monocytes (macrophages) or polymorphonuclear granulocytes, which include neutrophils, eosinophils, and basophils.

1.5.1 Lymphoid Cells

All lymphoid cells originate during hematopoiesis from a common lymphoid progenitor in the bone marrow. Their formation is known as lymphopoiesis. B cells mature in the bone marrow, while T cells mature in the thymus. The bone marrow and the thymus are called primary lymphoid organs. This is followed by migration via circulation into the secondary lymphoid tissue (spleen, lymph nodes, tonsils, and unencapsulated lymphoid tissue). The average human adult has about 10^{12} lymphoid cells, and lymphoid tissue as a whole represents about 2 % of the total body weight. Lymphoid cells represent about 20 % of the total white blood cells present in the adult circulation. After culmination of the immune response, many mature lymphoid cells live a very long life as memory cells.

Morphology Lymphocytes possess a large nucleus with little to no basophilic cytoplasm. Differences are seen in the nuclear (N) to cytoplasmic ratio, the degree of cytoplasmic staining with histological dyes, and the presence or absence of azurophilic granules.

Markers Most of the lymphocytes express specific cell surface makers on their cell surface. Some are present for a short duration, while others are responsible for their characterization. Such molecules can be used to distinguish various cell subsets. The selected antigenic markers are depicted in Table 1.2.

1.5.1.1 B Cells

These lymphocytes (Fig. 1.5) are unique due to their ability to secrete immunoglobulins. The word "B" refers to "bursa of Fabricius," an organ where B cells mature in birds. In humans, B cells are produced in the bone marrow. The development of B cells occurs at various stages, which include progenitor B cells, early pro-B cells, late pro-B cells, large pre-B cells, small pre-B cells, immature B cells, and mature B cells. Each stage is characterized by rearrangement of certain genes and expression of receptors. In particular, immature B cells start to express IgM receptors and mature B cells express IgD receptors as well.

The cell surface of each receptor possesses unique receptors called B-cell receptors (BCR), which have a membrane-bound immunoglobulin and will bind to a particular antigen. Following encounters with an antigen and recognition of a second signal from TH cells, B cells differentiate into plasma cells. Memory B cells are also formed from activated B cells, which are antigen specific, and quickly respond when they encounter the same antigen a second time, producing secondary immune response. All antigens do not require a second signal from TH cells to activate B cells, which is termed T-independent activation.

A single B cell has approximately 1.5×10^5 antibody molecules on its cell surface, which are all specific for a particular antigen. Other molecules expressed on mature B cells include B220, MHC class II molecules, CD21, CD32, CD35, CD40, CD80, and CD86. B220 is a form of $CD45^-$ $CD45R^-$ and is used to identify B cells despite not being exclusive for B cells. CD40 interacts with its ligand on TH cells, and this interaction is crucial for the development of B cells to differentiate into either antibody-secreting cells or memory cells.

After recognizing antigens through membrane-bound antibodies, there is B-cell proliferation and differentiation for about 4–5 days. This results in the production of plasma and memory cells. One of the five classes of antibodies is produced and secreted by plasma cells, which do not possess membrane-bound antibodies. Plasma cells survive for about 1–2 weeks.

B cells can be activated by both T-cell-dependent and T-cell-independent manner. For T-cell-dependent activation, a certain subset of effector T cells produced in response to an antigen can induce B cells, through a mechanism identified as immunologic synapse. Most antigens are T-cell dependent suggesting that maximal antibody synthesis depends on T-cell help. For T-cell-dependent antigen, the initial signal is emitted by cross-linking of the antigen to the B-cell receptor. The costimulation resulting from T cells constitutes the second signal. T-cell-dependent antigens are peptides, which are present on top of the MHC class II molecules of B cells and are presented to either TH2 cells or follicular helper T cells. The B cell presents the

Table 1.2 Selected antigenic markers on leukocytes

Antigen	Molecular weight (kD)	Distribution	Function
CD1	43–49	Dendritic cells, B cells	T-cell response
CD2	45–58	T cells, NK cells	T-cell activation
CD3	20–28	T cells, NKT cells	TCR expression and signal transduction
CD4	55	T cells, NKT cells	MHC class II-restricted immune recognition
CD5	58	T cells, B cells	Modulation of TCR and BCR signaling
CD8	32–34 each monomer α and β	T cells, TC class I-restricted T cells	Co-receptor
CD11a	180	Leukocytes	α chain of LFA-1-(adhesion molecule)
CD11b	160	Monocytes, granulocytes	α chain of complement receptor CR3 (MAC-1)
CD11c	150	Monocytes, granulocytes	α chain of p150, 95 (complement receptor/adhesion molecule)
CD16	50–80	NK cells, macrophages, neutrophils	Fc receptor subunit (low affinity). Phagocytosis, AD-antigen and ADC-cytotoxicity
CD21	130 (soluble) 145 (membrane bound)	B cells, follicular dendritic cells	Receptors for various antigens. Involved in signal transduction
CD22	140	Mature B cells	Adhesion and signaling
CD23	45	B cells, follicular dendritic cells, monocytes	IgE synthesis regulation, induction of inflammatory cytokines
CD25	55	Mitogen-induced T cells, monocytes/macrophages. Anti IgM-induced B cells	α subunit of IL-2 receptor
CD28	90	Most peripheral T cells. $CD3^+$ thymocytes	Co-stimulator for T-cell activation
CD29	130	Leukocytes	β subunit of VLA-1 integrin
CD32	40	B cells, monocytes, granulocytes	IgG molecule (Fc region) antigen-binding receptor
CD40	48 monomer	B-lineage cells, follicular dendritic cells. Endothelial cells, macrophages	B-cell growth, differentiation, isotype switching. Induction of cytokine release and adhesion molecules
CD45	180–220	Hematopoietic cells	T- and B-cell activation
CD45R	220, 205	B cells, T-cell subsets, granulocytes, monocytes	Restricted leukocyte common antigen
CD80	60	Activated B and T cells, macrophages	Co-stimulator of T-cell activation
CD86	80	B cells, monocytes, dendritic cells	Co-stimulator for T-cell activation

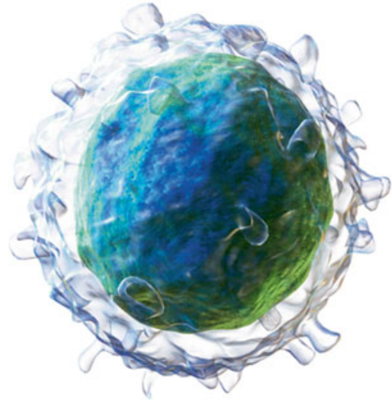

Fig. 1.5 Structure of a B lymphocyte (Source: Blausen.com staff. "Blausen gallery 2014." *Wikiversity Journal of Medicine*. doi:10.15347/wjm/2014.010. ISSN 20018762. – Own work. Licensed under CC BY 3.0 via Wikimedia Commons)

same antigen to the primed TH cells. As a consequence, T cells secrete cytokines that cause the proliferation and differentiation of B cells into plasma cells. Some cytokines cause class switch recombination (isotype switching).

The T-cell-independent B-cell activation involves type 1 T-cell-independent activation and type 2 T-cell-independent activation. T-cell-independent activation of B cells is rapid, but does not involve class switch recombination and germinal center formation. Germinal centers are located within secondary lymphoid organs. At this site, mature B lymphocytes divide, differentiate, and undergo mutation. For type 1, T-cell-independent activation takes place after an antigen is bound to B cells, and secondary activation is achieved through Toll-like receptors. The B cell produced is IgM restricted and is specific for the TLR-binding antigen. The expression of antigens on the surface of invading pathogen is in an organized and repetitive state, which forms the basis of type 2 T-cell activation. These antigens induce specific B cells through cross-linking of antigen receptors in various ways.

1.5.1.2 T Cells

T lymphocytes (Fig. 1.6) develop in the thymus and are consequently called T cells. However, their precursors are found in the bone marrow. The presence of the T-cell receptor distinguishes T cells from other lymphoid cells. There are presently two defined types of TCR; TCR-2 is a heterodimer of two disulfide-linked polypeptides with a molecular weight of 90,000 kD (α and β), and TCR-1 is structurally similar but consists of γ and δ polypeptide. Each chain has a constant and a variable region. These chains are characterized by an intrachain disulfide bridge and some sequence homology with the immunoglobulin domains. Both receptors are associated with a complex of polypeptides making up the CD3 complex. Thus, a T cell is defined

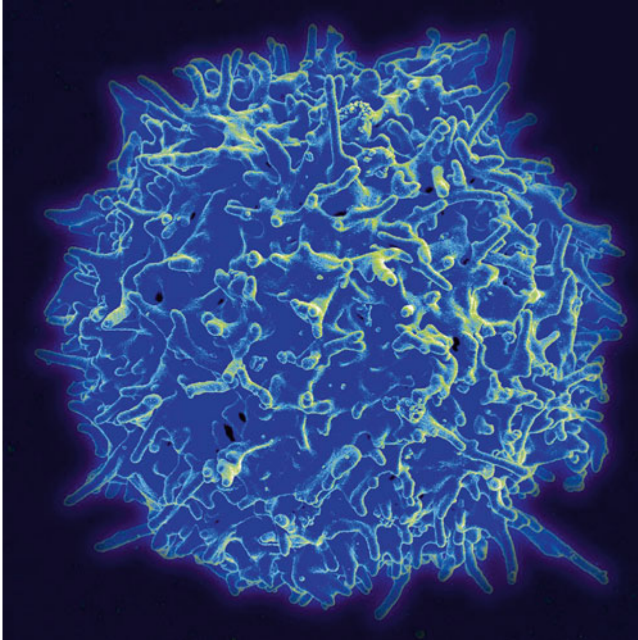

Fig. 1.6 Structure of a T lymphocyte (Source: NIAID, public domain)

either by TCR-1 or TCR-2, which is associated with CD3. Approximately 95 % of blood T cells express TCR-2 and up to 5 % have TCR-1. The TCR-2 cells are composed of two glycoprotein chains α and β and are subdivided into two distinct populations, the TH cells that are $CD4^+$ and Tc cells, which are $CD8^+$. $CD4^+$ T cells recognize antigens in association with major histocompatibility complex (HC) class II molecules, whereas $CD8^+$ T cells recognize antigens in association with MHC class I molecules.

Helper T Cells

Helper T cells ($CD4^+$ T cells) play the most crucial role in acquired immunity. The development of helper T cells takes place in the thymus, after positive and negative selection. In the thymus, cells carrying both $CD4^+$ and $CD8^+$ cells are eliminated, and only cells recognizing either MHC class I or MHC class II molecules are retained. The cells recognizing the antigen in the context of MHC class II molecules become the helper T cells. $CD4^+$ T cells are activated through class II-restricted antigens. They regulate a number of immune responses including T-cell proliferation, isotype switching of the antibodies, generation and effector function of cytolytic T cells, induction of NK cell activity, and inducing bactericidal activity of phagocytes. Most of their effects are mediated via secretion of cell-specific

cytokines. They are called $CD4^+$ T cells, as they express CD4 receptors. However, there are some other cells that also express CD4 receptors, but they are considered as exception to the rule. While there are two major subsets of helper T cells, TH1 and TH2, additional subsets including TH17, TH9, Treg, TH22, and THαβ have also been identified.

Induction of $CD4^+$ T cells results in their expansion and differentiation into effector as well as regulatory subsets. $CD4^+$ effector T cells, TH1 and TH17, and Treg cells function in metabolically different ways. In murine models, inflammatory effector T cells maintain enhanced levels of glycolytic genes and depend on high glycolytic rates, whereas Treg cells are oxidative for their division, differentiation, and survival. They also depend on the mitochondrial electrons. The bifurcation between T-cell glycolytic and oxidative metabolism depends on pyruvate dehydrogenase. Pyruvate dehydrogenase kinases suppress the activity of pyruvate dehydrogenase. Pyruvate dehydrogenase kinase 1 is present in TH17 cells, and in low amounts in Treg cells, but not in TH1 cells. The antagonism or deletion of pyruvate dehydrogenase kinase 1 specifically inhibits TH17 cells and augments Treg cells. It is apparent that $CD4^+$ subsets employ specific metabolic programs, and the inhibition of these pathways should regulate diverse T-cell subsets, which may have clinical implications.

The major two subsets of helper T cells are differentiated on the basis of cytokines that they secrete, as a result of their proliferation. TH1 cells are involved in cytotoxicity and regulate the killing of the invading pathogens, specifically intracellular bacteria and protozoa. They are characterized by the secretion of IFN-ϒ and are induced by IL-12, but they also secrete additional cytokines including TNF-α and TNF-β. The effector cells for TH1 immune response include cytolytic T cells, macrophages, IFN-ϒ, CD4 T cells, and IgG B cells. STAT4 and T-bet are the predominant transcription factors for TH1 cells. TH2 cells support B-cell activation, isotype switching, IgE production, recruitment, and induction of various cell types and are antiparasitic by natural design. This function is performed by TH2 cells through secreting a variety of cytokines, including IL-4, IL-5, IL-10, and IL-13. IL-2, IL-3, IL-25, IL-31, and GM-CSF are secreted by both subsets. The proliferation of TH2 cells is induced by IL-4. STAT6 and GATAs are the major transcription factors for TH2 cells. However, there is also IL-4-/STAT6-independent signaling, which may result in TH2 differentiation in a complementary way. Eosinophils, basophils, mast cells, B cells, and IL-4/IL-5 CD4 T cells are the principal effector cells. TH2 cells are predominantly involved in the etiology and pathogenesis of allergic disease and asthma.

TH17 is a subset of helper T cells that produces IL-17. These cells are responsible for establishing immunity to the microbial agents at epithelial/mucosal barriers (Fig. 1.7). TH17 cells develop separately from TH1 and TH2 cells. They are induced either by IL-6 and TGF-β or IL-23 and IL-1β. Their effector cells include neutrophils, IL-17 $CD4^+$ T cells, and IgM/IgA B cells. STAT3 and retinoic acid receptor-related orphan receptors G are their main transcription factors. The effector cytokines include IL-17, IL-21, and IL-22. Deficiency/lack of TH17 cells leaves a host susceptible to opportunistic infections. IL-22 acts on epithelial cells to produce certain proteins that are bactericidal. In response to distinct cytokines, TH17 cells

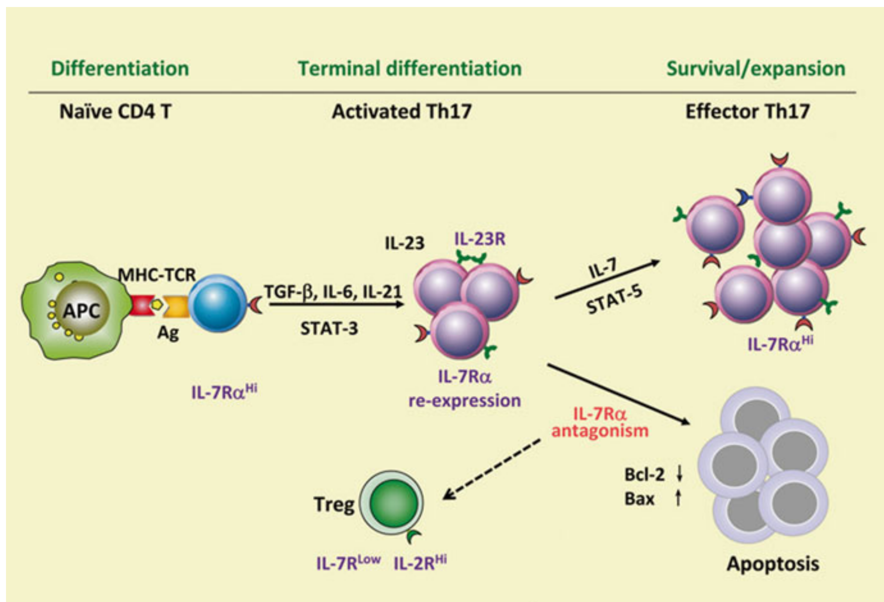

Fig. 1.7 This figure depicts the differentiation, survival, and proliferation of TH17 cells. The role played by IL-17 in the survival and proliferation of TH17 cells is also shown. Other cytokines including IL-6, IL-21, and IL-23 are also involved in the differentiation of TH17 cells. During T-cell activation and differentiation, IL-7 receptors are expressed on TH17 cells (Reproduced with permission, Source: Leung S, Liu X, et al. (2010). The cytokine milieu in the interplay of pathogenic TH1/TH17 cells and regulatory T cells in autoimmune disease. *Cellular and Molecular Immunology*, 7: 182-189. Nature Publishing Group)

will develop either into protective or pro-inflammatory cells. The pro-inflammatory TH17 cells are produced by IL-23 and IL-1 β, and the protective TH17 cells are generated by IL-6 and TGF-β, which are the T regulatory 17 cells.

TH17 cells are involved in the production of two members of IL-17 family. These members are IL-17A and IL-17F, which play a role in the recruitment, activation, and migration of neutrophils (Fig. 1.7).

The steroid receptor-type nuclear receptor RORϒt is required for IL-17 production, a cytokine released by TH17 cells. In addition to RORϒt, another member of the retinoid nuclear receptor family, RORα, which is also selectively expressed in TH17 cells, plays a similar but not identical role in the differentiation of TH17 cells. These observations suggest that two lineage-specific transcription factors are involved in the differentiation of TH17 cells. STAT3 plays a role in the induction of RORϒt. IL-6, IL-21, and IL-23 play a crucial role in this process via specifically activating STAT3.

The development of TH17 cells and Treg cells is interconnected reciprocally. This was demonstrated by experiments where TGF-β in the presence of either IL-6 or IL-21 resulted in the development of TH17 cells and in inhibition of Treg development. The presence of both TGF-β and IL-6 or IL-12 results in a strong

production of IL-17 cells from the naïve T cells. However, this response is not produced if the aforementioned combination is not used.

IL-23 also plays an important role in inducing the synthesis of IL-17 by activated T cells. A full sustained development of TH17 cells requires IL-23. For a productive and sustained TH17 response, the presence of IL-23 is required, despite the observation that IL-23 is not a differentiation factor for this T-cell subset. In contrast both IFN-ϒ and IL-4 are inhibitors of TH17 differentiation. TH17 cells are involved in inflammation and tissue injury in autoimmune diseases that include rheumatoid arthritis, Crohn's disease, juvenile diabetes, multiple sclerosis, autoimmune uveitis, and psoriasis.

TH9 cells were originally considered to be included in TH2 cells and they secrete IL-9. Since both IL-4 and IL-9 are rarely secreted by the same cell, IL-9-producing cells are classified as a separate subset of helper T cells. IL-9 is secreted by TH9 cells in response to IL-4, TGF-β, and IL-1 and inhibited by IFN-ϒ. The development of TH9 cells is also induced by other stimuli, which include IL-25, tumor necrosis factor receptor superfamily member 4 (TNFRSF4 or Ox40), cyclooxygenase (COX)-2, 1,25-dihydroxyvitamin D3, calcitonin gene-related peptide (CGRP), thymic stromal lymphopoietin (TSLP), Jagged 2, and programmed cell death ligand (PD-L)2. The development of TH9 cells is mediated by a series of complex pathways. IL-2 receptors, IL-4 receptors, and TGF-β-dependent signal transduction pathways are required for their development. The CD28-derived signals are important in the induction of IL-9 production by TH9 cells. Their development is transcriptionally regulated by IRF4 and PU.1. IL-9 plays a role in the development of allergic and autoimmune diseases. Furthermore, they may also play a role in cancer, since in animal models they augment immune response against melanoma (Fig. 1.8).

THαβ helper cells are responsible for providing immunity against viruses. They are induced by IFN-α/IFN-β and/or IL-10. The latter is the key effector cytokine for the THαβ helper subset. The main effector cells include NK cells, cytolytic T cells, IgG B cells, and IL-10 $CD4^+$ T cells. STAT1, STAT3, and IRFs are their principal transcriptional molecules.

TH22 cells are identified as a separate subset of helper T cells since some T cells secrete IL-22 independent of IL-17, specifically $CCR10^+$ T cells. IL-22 belongs to IL-10 family and binds to a heterodimer receptor (IL-10 receptor β chain and the IL-22R); its function is distinct when compared to IL-10. IL-22 is expressed only on nonimmune cells and also induces three MAPK pathways. The production of IL-22 is dependent on IL-23 and transcriptional factors T-bet and AhR in some experimental models. It is involved in epithelial innate responses with dual protective as well as detrimental roles. IL-22 is neither pro- or anti-inflammatory in its effects. The distinct function of TH22 cells is epidermal immunity and remodeling.

Cytolytic T Cells

Cytolytic T cells ($CD8^+$ T cells) kill virus-infected and cancer cells. They are antigen specific and recognize their antigen in the context of MHC class I molecules. The development of cytolytic T cells takes place in the thymus, after positive and

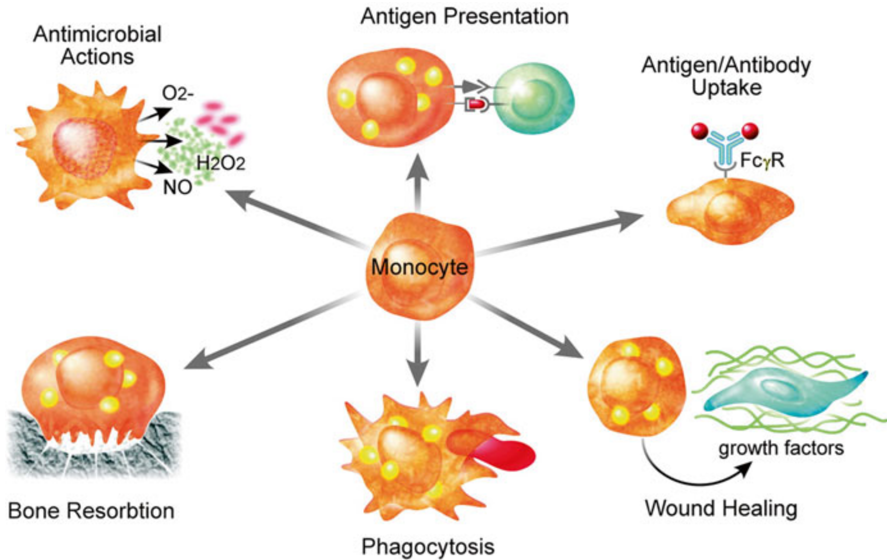

Fig. 1.8 This figure depicts the development and role of TH9 cells. TH9 cells develop from naïve TH cells requiring IL-2 and are induced by TGF-β and IL-4. Their production is augmented by IL-1, IL-5, IL-7, IL-8, and IL-9. TH9 cells via releasing IL-9 play a role in allergic disease, autoimmune disorders, leukemia, and melanoma (Reproduced with permission, Source: Schmitt et al. (2014) Th9 cells, a new player in adaptive immunity. *Trends in Immunology*. 35: 61–68. Elsevier Limited)

negative selection, where the cells carrying both $CD4^+$ and $CD8^+$ cells are eliminated, and only cells recognizing either MHC class I or MHC class II molecules are retained. The cells recognizing antigens in the presence of MHC class I molecules become the cytolytic T cells. They are activated through class I-restricted antigens. After the development into effector cytolytic T lymphocytes, they migrate to the tissues where they are activated in response to coming in contact with the target cells. Upon activation cytolytic T cells will proliferate in response to IL-2. Cytolytic T cells release perforin, granulysin, and granzymes, which are all cytotoxins. These cytolytic effector proteins are present in cytoplasmic exocytic granules. Granzyme, a part of large family of serine proteases, is expressed in a variety of cells involved in innate and acquired immunity. It enters the cytoplasm of the target cells through perforin-mediated entry, and a caspase cascade is activated. Perforin is a pore-forming protein, and these series of events lead to apoptosis by chymase activity of granzyme, of the target cells. In animal models, inactivation of Prf1 gene inhibits cytolytic T-cell effector function and immune response, without compromising the development of T lymphocytes.

The killing of target cells by cytolytic T cells requires TCR, MHC class I molecules, and interaction of adhesion molecules. In particular, interaction of LFA-1 expressed on cytolytic T cells and ICAM molecules expressed on the target cells plays a stabilizing role in this effector function. The TCR-/CD3-associated signal transduction activates tyrosine kinase that causes phosphorylation of PLCϒ, activa-

tion of PKCs, and Ca^{++} influx. As a result there is granule polarization and exocytosis, and after contact cytolytic T cells release their content into the target cell resulting in its killing.

Natural Killer T Cells

Activation of natural killer T cells results in the performance of functions similar to $CD4^+$ and $CD8^+$ T cells. They release cytokines including IL-4, IFN-γ, GM-CSF, as well as others and are also cytotoxic. They are mostly not MHC restricted, instead they recognize antigens that are glycolipids and require CD1d. NKT cells bridge innate and acquired immune responses. They express both αβ T-cell receptor and markers present on natural killer cells such as NK1.1. Natural killer T cells include $NK1.1^+$, $NK1.1^-$, $CD4^+$, $CD8^+$, $CD4^-$, and $CD8^-$ cells. They also express CD16 and CD56 antigens, which are present usually on NK cells. NKT cells are classified into type 1 NKT, type 2 NKT, and NKT-like cells. NKT-like cells are not CD1d restricted and may have MHC or other restrictions. Abnormal NKT cell function may be associated with cancer and autoimmune disease.

Gamma Delta T Cells (ϒδ T Cells)

Gamma delta T cells are a minor subset of T cells that express a distinct T-cell receptor on their surface membrane. Their distribution is high in gut mucosa, included in a population of lymphocytes known as intraepithelial lymphocytes (IELS). This subset has a T-cell receptor, which is made up of one ϒ and one δ chain. In the peripheral blood, the γδ T-cell population is composed of Vγ9/Vδ2 T cells and is unique in the sense that this is specific for a non-peptide microbial metabolite, HMB-PP, which is a precursor of isopentenyl pyrophosphate. The γδ T-cell population produces a rapid response after recognizing HMB-PP. They do not recognize peptide epitopes in the context of MHC molecules after antigen uptake and processing. However, some recognize MHC class Ib molecules. They play a prominent role in the recognition of lipid molecules. Gamma delta T cells are of invariant nature, and perhaps alarm signals such as heat shock proteins cause their induction. In addition, there is a subpopulation of this T-cell subset present in the epidermal compartment of the skin (murine) and termed dendritic epidermal T cells.

They are considered “first-line defense” or a bridge between innate and acquired immune responses. Gamma delta T cells develop in the thymus and in the periphery, in response to signals from other leukocytes. They divide into functionally distinct subsets, after maturation and influence immune cells, as well as healthy tissue, both directly and indirectly. Gamma delta T cells produce host responses to the invading pathogens. Their features classify them in between innate and acquired response, because on one hand they allow a rapid immune response against a foreign antigen and on the other hand rearrange TCR genes to cause junctional diversity. The later function is a part of acquired immunity due to the development of a memory phenotype in response to an antigen. But their restricted TCR may be utilized as a pattern

recognition receptor, and thus they will be classified as part of innate immune response. Based on additional available data, it seems that gamma delta T cells can fit the definitions of both innate and acquired immune responses.

Memory T Cells

Memory T cells belong to a subset of infection and cancer-fighting T cells, which have previously recognized and responded to their cognate antigen. They recognize viruses, bacteria, or cancer cells. Memory T cells are a product of primary immune response, and as a consequence, when they face the same invading pathogen or the tumor cell for the second time, they produce a rapid and potent response. Three distinct subsets of memory T cells have been found, as they can be recognized by the differential expression of L-selectin and CCR7. The first subset is called stem memory T_{scm} cells. They are $CCR7^+$, $CD45RO^-$, $CD62L^+$, $CD45RA^+$, $CD27^+$, $CD28^+$, and IL-$7R\alpha^+$. The second subset is called central memory T_{cm} cells. They secrete IL-2, but not IL-4 or IFN-ϒ, and express CCR7 and L-selectin. The third subset is called effector memory T_{EM} cells. These cells produce IL-4 and IFN-ϒ, but do not express CCR7 or L-selectin. T_{CM} and T_{EM} subsets contain antigen-specific memory T cells directed against viruses and other microbial molecules. Both helper and cytolytic T cells contain these subsets of memory T cells. The T_{CM} has some common characteristics, which is also exhibited by memory cell stem cells. There is an increased level of phosphorylation of STAT5 in these cells, which allows their self-renewal. As opposed to T_{EM} cells, the T_{CM} cells are more potent in conferring immunity against viruses, bacteria, and tumor cells.

Memory B cells are produced within germinal centers after the primary infection. They are responsible for a rapid and robust secondary immune response, following the reexposure to their specific primary antigen. During primary immune response or first exposure to an invading pathogen exhibiting T-dependent antigen, in the presence of T_{FH} cells within the follicles of secondary lymphoid organs, there is an activation of naïve follicular B cells. This results in the production of antigen-specific foci of B cells. These cells differentiate to become antibody-secreting cells (plasma cells) to fight infection. After the infection is cleared, a fraction of these cells remain as dormitory memory cells. These memory cells have long life, after they go through a mutative and selective germinal center reaction. The activated B cells that do not go through germinal center differentiation are eliminated. With each subsequent exposure to the same antigen, there is a generation of polyclonal secondary response, and increased number of memory B cells remains.

Regulatory T Cells

The Treg cells were previously known as the suppressor T cells and are pivotal in maintaining immune tolerance. Their principal functions include a negative feedback after the generation of an immune response to limit unintended damage and to protect from autoimmunity. These cells are described in detail in chapter 10.

1.5.2 Natural Killer Cells

After B and T cells, NK cells are the third largest class of lymphocytes that were originally identified due to their spontaneous killing ability of tumor cells. They develop in the bone marrow from common lymphoid progenitor and require IL-15, C-KIT, and FLT-3. NK cells share effector function and the ability to produce cytokines with T cells. They were previously referred to as null cells because they do not express either T-cell or B-cell receptors, do not secrete antibodies, and do not possess antigen-recognizing receptors. Their morphology is also different from B and T cells, as they are large granular lymphocytes. These cells make up about 5–10 % of the lymphocytes in human peripheral blood.

NK cells participate in innate immunity and are the first responders against infection and possibly tumors. They are $CD3^-$ and also lack immunoglobulin receptors; however, they carry CD56 antigen, which is used to identify these lymphocytes. NK cells also express CD16, which is a low-affinity receptor for IgG and is not present on mature T cells. They are involved in antibody-mediated cellular cytotoxicity and apoptosis. Following binding of CD16 of NK cells to the Fc portion of IgG, cytoplasmic granules are released, which cause the destruction of the target cell where CD16 facilitates the release of the cytoplasmic granules. Natural cytotoxicity receptors (NCR) are exclusively expressed on NK cells and include NKp30, NKp44, NKp46, as well as NKp80. NKp44 and NKp46 are important in defense against viruses, as they bind to influenza hemagglutinin. Another NK cell surface receptor, 2B4, binds to CD48 and may play a role in the defense against Epstein–Barr virus. A defect in 2B4 function is associated with the X-linked lymphoproliferative syndrome. In addition, NK cells possess receptors for a variety of cytokines including IL-2, IL-12, IL-15, IL-21, IFN-α, and IFN-ϒ.

The killing ability of NK cells is associated with the expression of MHC class I molecules. According to the "missing-self hypothesis," NK cells search for the presence of MHC class I molecules, which are ubiquitously expressed. A decrease in the expression of MHC class I molecules on a cell allows NK cells to kill the target, as it is released from the influence of MHC class I molecules. The ability of NK cells to kill tumors and virally infected cells resides in several inhibitory receptors called immunoglobulin-like receptors and CD94/NKG2 heterodimers. The immunoglobulin-like receptors include KIR, immunoglobulin-like transcripts (ILT), and leukocyte Ig-like receptors (LIR). These receptors bind to HLA and after a cascade of signal transduction, inhibit NK cell stimulation. In addition to these inhibitory receptors, there are also stimulatory receptors that rely on perforin and INF-γ for their function. NK cells produce a number of other cytokines and chemokines, including TNF-α, IL-5, IL-13, GM-CSF, MIP-1 (α and β), as well as RANTES.

These cells are crucial in fighting viral infections as part of early innate response. Severe systemic viral infections, specifically herpes virus, may result from a lack or malfunction of NK cells. Patients infected with HIV have low numbers of NK cells. They also play a role in killing tumors. The patients with Chediak–Higashi syndrome have an increased risk of lymphomas, and this disease is associated with

impaired NK cells, macrophages, as well as neutrophils. A number of cytokines including IL-2, IL-12, IL-15, IL-21, IFN-α, and IFN-β induce NK cells, which results in their proliferation, margination, cytokine production, and cytotoxicity.

1.5.3 Antigen-Presenting Cells

APCs are a heterogeneous population of cells with extraordinary immunostimulatory capacity. Some play an important role in the induction of the function of the activity of helper T cells, and some communicate with other lymphocytes. Cytokines can render the ability to present antigens to a variety of cell types. This results in the expression of MHC class II molecules, which are sometimes lacking on some cells such as endothelial cells. The different types of antigen-presenting cells include macrophages, dendritic cells, B cells, and interdigitating cells. Antigen-presenting cells are mostly derived from the bone marrow and are distributed in lymphoid tissues as well as in the skin. These three types of major antigen-presenting cells are also called professional antigen-presenting cells.

1.5.3.1 Macrophages

Macrophages (Fig. 1.9) participate in innate as well as acquired immune response. As opposed to T and B cells, they are not characterized by any specific cell surface receptors and play an important role in normal tissue repair and aging. They are phagocytes that continuously remove self-proteins, which are degraded and presented to T cells in the context of MHC class II molecules. However, this does not result in the activation of T cells, since in the absence of infection, the expression of MHC class II molecules on macrophages is low and the presence of B7, a costimulatory molecule, is almost negligible. Following infection, there is an upregulation of MHC class II and B7 molecules.

Also termed as mononuclear phagocytic system, monocytes circulate in the blood and macrophages in the tissues. In the bone marrow during hematopoiesis, the progenitor cells for granulocytes–monocytes differentiate into promonocytes. The promonocytes then leave the bone marrow and enter the bloodstream. In the blood, promonocytes mature into monocytes. Monocytes/macrophages are derived from the bone marrow stem cells. After monocytes enter damaged tissue via endothelium by chemotaxis, they differentiate into macrophages. Monocytes undergo multiple changes during differentiation to macrophages; they enlarge several folds, the number of intracellular organelles is increased, they are able to hydrolyze enzymes, and their phagocytic ability is augmented. As shown in Table 1.3, macrophages are classified according to their tissue distribution, where their functions are diverse and tissue specific.

The most important role of macrophages is antigen presentation. However, in addition to antigen presentation, macrophages play several other important roles in

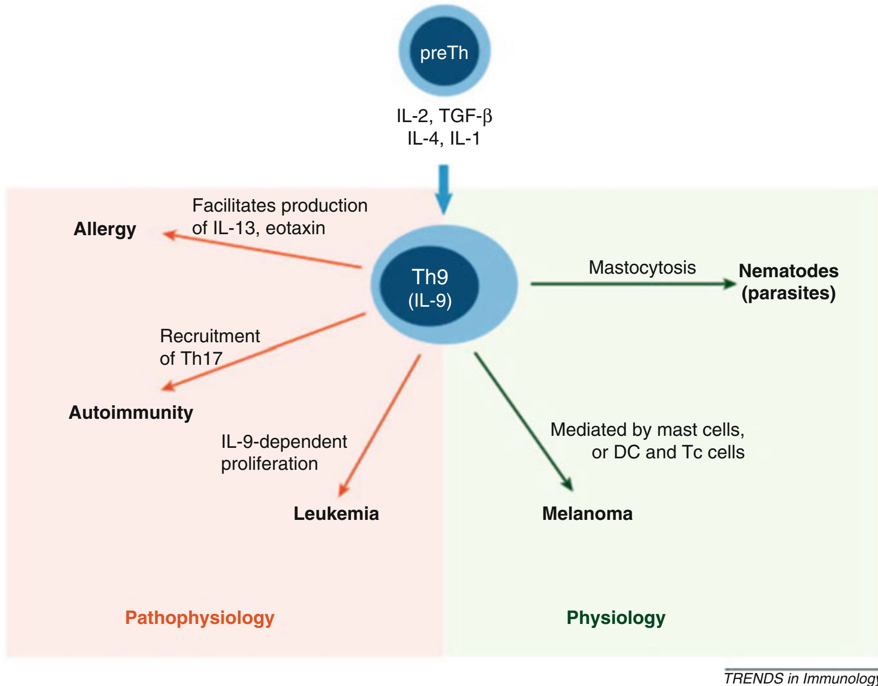

Fig. 1.9 Functions of monocytes/macrophages: macrophages are involved in first line of defense against invading pathogens. This is performed by producing oxidative burst and release of the inflammatory mediators. They are also involved in acquired immune response through their role in antigen presentation and cytokine secretion. They clear immune complexes and cause wound healing by releasing growth factors (Source: Chawla A. *Circulation research*, 2010, 106: 1559–1569, Lippincott Williams & Wilkins, reproduced with permission)

Table 1.3 The types of macrophages and their distribution

Name	Tissue type
Alveolar macrophages	Lungs
Kupffer cells	Liver
Histiocytes	Connective tissue
Microglial cells	Brain
Osteoclasts	Bone
Mesangial cells	Kidney

immune response, which include inflammatory response, antitumor activity, microbicidal activity, lymphocyte activation, and tissue reorganization. Most of their physiopathological effects are mediated via cytokines. In addition to the microbicidal activity, they release oxygen-dependent free radicals, cytotoxins, antimicrobials, and oxygen-independent hydrolases. For tissue reorganization, they secrete collagenases, elastases, and angiogenesis factors (Fig. 1.9).

Oxygen-dependent killing mechanisms involve the production of a number of reactive oxygen and nitrogen intermediates, which possess potent antibacterial activity, by the activated macrophages. The reactive oxygen intermediates are produced as a result of a process called the respiratory burst and include superoxide anion (O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and hypochlorite anion (CLO). The reactive nitrogen intermediates include nitric oxide (NO), nitrogen dioxide (NO_2), and nitrous acid (HNO_2). Oxygen-independent killing involves TNF-α, defensins, lysozymes, and hydrolytic enzymes.

1.5.3.2 Dendritic Cells

Dendritic cells (Fig. 1.10) are very important antigen-presenting cells along with mononuclear phagocytes. They are distributed in small quantities in various tissues that come in contact with the external environment, including the skin and the inner lining of the nose, lungs, stomach, and intestine. The blood contains immature dendritic cells. After activation, dendritic cells migrate to the lymphoid tissue to initiate an acquired response following their interaction with lymphoid cells, B cells, and T cells. There are two most common types of dendritic cells, myeloid dendritic cells and lymphoid dendritic cells. Myeloid dendritic cells secrete IL-12, are very similar to monocytes, and can be divided into at least two subsets, mDC1 and mDC2. mDC1 stimulates T cells, whereas mDC2 may have a role in fighting wound infection. The lymphoid dendritic cells are similar to plasma cells and produce high levels of interferon-α. The dendritic cells are of hematopoietic origin, having myeloid dendritic cells of myeloid origin and lymphoid dendritic cells of lymphoid origin. The types and distribution of dendritic cells are shown in Table 1.4.

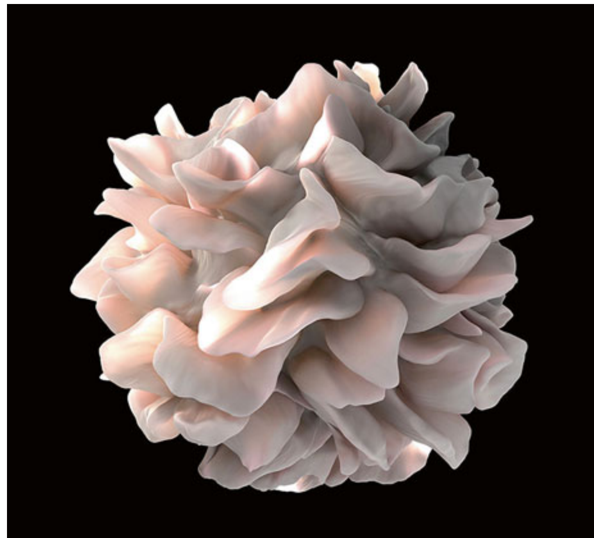

Fig. 1.10 Artistic dawning of the surface of dendritic cells (Source: NIH, Public domain)

Table 1.4 Classification of dendritic cells

Type	Distribution
Circulating dendritic cells	Blood, lymph
Langerhans cells	Skin, mucous membrane
Interdigitating dendritic cells	Secondary lymphoid tissue (with T cells), thymus
Interstitial dendritic cells	Gastrointestinal tract, heart, liver, lungs, kidney

Table 1.5 Features of immature dendritic cells

Presence of CD1a molecules on cell surface
Augmented expression of MHC class II molecules
A lack or very low expression of costimulatory molecules
IL-10 inhibits maturation
A lack or very low expression of CD25, CD83, and p55

The skin dendritic cells are called Langerhans cells, are of myeloid origin, and are $CD34^+$ with high levels of HLA-DR. The hematopoietic progenitors for other myeloid dendritic cells are also $CD34^+$. Furthermore, they are derived from cells of either $CD14^+CD1a^-$ or $CD14^-CD1a^+$ lineage. The dendritic cells derived from $CD14^+CD1a^-$ are interstitial and/or peripheral blood dendritic cells with phagocytic properties. Their maturation is induced by IL-4, IL-13, GM-CSF, and TNF. The dendritic cells derived from $CD14^-CD1a^+$ are epidermal dendritic cells and their maturation is induced by TGF-β.

The progenitor cells for dendritic cells are present in the bone marrow, which initially transform into immature dendritic cells (Table 1.5). The presence of Toll-like receptors on these cells, mDC (TLR2, TLR4), lymphoid dendritic cells (TLR7, TLR9), along with other receptors, collectively called as pattern recognition receptors (PRRs), allows the immature dendritic cells to search for viruses and bacteria in their environment. A contact with an invading pathogen allows these cells to quickly develop into mature dendritic cells. Although immature dendritic cells can process antigens, only mature dendritic cells can present the antigenic fragment in the context of MHC molecule to T cells. During T-cell activation, a number of co-receptors including CD40, CD80, and CD86 are simultaneously upregulated by dendritic cells. A chemokine receptor, CCR7, which allows migration of dendritic cells from blood to secondary lymphoid organs, is also upregulated by these cells. Dendritic cells communicate with other cells by cell–cell interaction. For example, CD40L present on lymphocyte binds to CD40 receptors on dendritic cells, resulting in cross talk. Dendritic cells promote the generation of TH1 cells via IL-12 production after they come in contact with a pathogen. The characteristics of mature and immature dendritic cells are further pointed out in Tables 1.5 and 1.6.

Lymphoid dendritic cells promote negative selection in the thymus. This may be attributed to their ability to induce Fas-mediated apoptosis. Based on their ability to cause apoptosis and their ability to eliminate self-reactive T cells, lymphoid dendritic cells exhibit a regulatory function instead of a stimulatory immune effector function. Myeloid dendritic cells also have differential effects. For example, T cells can be primed to selectively activate TH1 responses by CD14-derived myeloid den-

Table 1.6 Features of mature dendritic cells

Antigen is uptaken by macrophage mannose receptor and DEC-205 receptors
Present antigen in the context of MHC class I and class II molecules
T-cell binding and costimulatory molecules including CD40, CD54, CD58, CD80, and CD86 have ample expression
Macrophage-restricted molecules are not present. Produce IL-12 in large quantities
Resistant to p55, CD83, S100b, IL-10 dendritic cells – restricted molecules

Table 1.7 Characteristics of major antigen-presenting cells

	Macrophages	B cells	Dendritic cells
Distribution	Lymphoid and connective tissues. Body cavities	Blood. Lymphoid tissue	Skin. Lymphoid and connective tissue
Antigens expressed	MHC class I, low levels of MHC class II (inducible with stimulus), low levels of B7 (inducible with stimulus)	MHC class I, MHC class II (further induced by stimulus). Low levels of B7 (inducible by stimulus)	MHC class I, MHC class II, high expression of B7
Types of antigens presented	Extracellular antigen in the context of MHC class II molecules	Extracellular antigens. Presentation involves immunoglobulin receptors and MHC class II molecules	Extracellular and intracellular antigens in the context of MHC class I and II molecules

dritic cells. Naïve B cells can be activated in the presence of CD40L and IL-2 to secrete IgM by $CD34^+$, CD14-derived myeloid dendritic cells. This effect on naïve B cells is not observed with CD1a-derived dendritic cells.

1.5.3.3 Nonprofessional Antigen-Presenting Cells

In addition to the three major classes of antigen-presenting cells (Table 1.7) already described, a number of other cells including fibroblasts, thyroid epithelial cells, thymic epithelial cells, glial cells, endothelial cells (vascular), and pancreatic β cells can be induced to present antigens to T cells. These cells do not constitutively express MHC class II molecules, but after their exposure to certain cytokines such as INF-γ, the MHC molecules are expressed, which enables them to present antigen to naïve T cells.

1.5.4 Polymorphonuclear Leukocytes

1.5.4.1 Neutrophils

Approximately 90 % of circulating granulocytes are neutrophils (Fig. 1.11). They possess a multilobed nucleus and a granulated cytoplasm. Neutrophils are produced in the bone marrow by hematopoiesis and then migrate to the bloodstream. They are terminally differentiated, and after entering into the tissues, they live for only a few

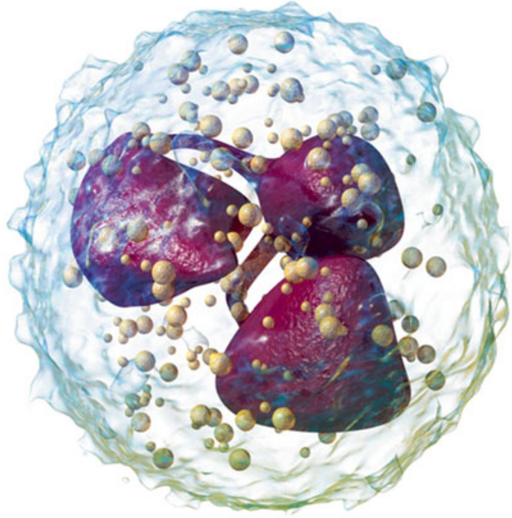

Fig. 1.11 Three-dimensional structure of a neutrophil (Source: Blausen.com staff. "Blausen gallery 2014" *Wikiversity Journal of Medicine*)

days. However, their lifespan can be increased in the presence of IL-2 as they express IL-2βR. At the site of injury/inflammation, neutrophils appear first, and as a result of infection, their production and release is augmented in the bone marrow. The migration of neutrophils from the blood to the tissues is regulated by adhesion molecules, which will be described later in this chapter. Briefly, the neutrophils first bind to vascular endothelium that allows their transport into the tissue by the creation of gaps in the blood vessels. The path of their migration is directed by various molecules including IL-8, IFN-γ, and C5a, which bind to neutrophils via their specific receptors that are present on their cell surface membrane. This whole process is called chemotaxis. The gathering of neutrophils at the site of infection/injury/inflammation is further aided by the production of a variety of chemotactic factors.

Neutrophils are phagocytes as is the case for macrophages. They employ a number of bactericidal substances and lytic enzymes in addition to both oxygen-dependent and oxygen-independent pathways to kill microbes. Three types of granules, primary granules, secondary granules, and tertiary granules, contain a variety of proteins that are released as a result of stimulus-induced degranulation. The primary granules, which are termed as azurophilic granules, contain myeloperoxidase, defensins, cathepsin G, and bactericidal/permeability-increasing protein. The secondary granules (specific granules) contain lactoferrin and cathelicidin, and the tertiary granules contain gelatinase and cathepsin. Neutrophils can regulate the function of monocytes and lymphoid cells via a variety of cytokines including IL-1β, IL-1ra, IL-8, TGF-β, TNF-α, as well as preformed granules. The production of cytokines by neutrophils is variable depending on the stimulus.

1.5.4.2 Eosinophils

Eosinophils (Fig. 1.12) develop in the bone marrow where they also mature. They are then released into the blood followed by their migration into the tissue spaces. The eosinophils comprise 1–5 % of blood leukocytes in nonatopic individuals. The eosinophils are present in the thymus, spleen, lymph nodes, uterus, and lower gastrointestinal tract. Under normal conditions, they are not present in the skin, lungs, or esophagus. They can live up to 72 h in the tissue. Their migration to the site of inflammation or parasitic infection is directed by leukotriene B4 and chemokines eotaxin (CCL11) as well as RANTES (CCL5). They play a specialized role in immunity to helminth infection by releasing special granules. Eosinophils are also capable of phagocytosis and killing ingested microorganisms, but this is not their primary function.

Their immune function is mediated via production of cationic granule proteins, reactive oxygen species, leukotrienes, prostaglandins, elastase, a plethora of cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF-α), and growth factors (PDGF, TGF-β, VEGF). The cytotoxic granules released by eosinophils in response to a stimulus include major basic protein, eosinophil cation protein, eosinophil peroxidase, and eosinophil-derived neurotoxin.

Eosinophils are attracted by proteins released by T cells, mast cells, and basophils (eosinophil chemotactic factor of anaphylaxis, ECF-A). They bind schistosomulae coated with IgG or IgE, degranulate, and release major basic protein, which is toxic. Eosinophils also release histaminase and arylsulphatase, which inactivates histamine and SRA-A. This results in anti-inflammatory effects and inhibits migration to granulocytes to the site of injury.

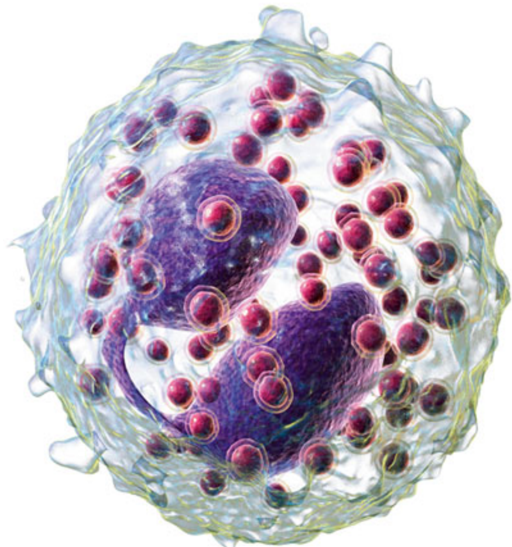

Fig. 1.12 Three-dimensional structure of eosinophils (Source: Blausen.com staff. “Blausen gallery 2014” *Wikiversity Journal of Medicine*)

1.5.4.3 Basophils and Mast Cells

Basophils are found in very small numbers in the circulation (0.01–0.3 % of leukocytes) and are non-phagocytic granulocytes. They originate in the bone marrow and contain large cytoplasmic granules. After activation, basophils secrete a number of mediators including histamine, leukotrienes, proteoglycans, and proteolytic enzymes, as well as several cytokines. These substances play a pivotal role in allergic responses.

Mast cells are similar to basophils, but they are derived from different precursor cells in the bone marrow. The precursor cells for both basophils and mast cells express CD34. Mast cells are released from the bone marrow in an undifferentiated state into the blood, and they differentiate upon reaching the tissue. Mast cells are distributed in a wide variety of tissues including the skin, lungs, digestive tract, genitourinary tract, nose, and mouth. There are two types of mast cells, the connective tissue mast cells (CTMC) and mucosal mast cells (MMC). The activity of mucosal mast cells is dependent on T cells. Mast cells play a crucial role in allergic disease and inflammation. They express receptors for IgE (FcεR1), and binding of the antibody to its receptor followed by a second exposure to the specific antigen results in massive degranulation of mast cells. The mediators released by mast cells include histamine, prostaglandin D2, leukotriene C4, heparin, serine proteases, and a plethora of cytokines. In addition to their role in allergic disease and anaphylactic shock, mast cells are implicated in autoimmune diseases, including rheumatoid arthritis and multiple sclerosis, as well as induction of peripheral tolerance. Mast cells also participate actively in the innate immune responses to many pathogens. They play a role as innate effector cells in augmenting the initial events in the development of adaptive immune responses. Mast cells may also play a role in combating viral infections through various direct or indirect pathways. These pathways involve the activation of mast cells by pathogens including Toll-like receptors and co-receptors.

1.6 Molecules Which Recognize Antigen

The acquired immune response is designed to recognize foreign antigens, which is achieved by two distinct types of molecules that are involved in this process: the immunoglobulins and the T-cell antigen receptors. The immunoglobulins or antibodies are a group of glycoproteins present in a number of bodily fluids including the serum and are secreted in large amounts by plasma cells that have differentiated from precursor B cells. B lymphocytes possess membrane-bound immunoglobulins, which have similar binding specificity as that of the terminally differentiated plasma cells. Recognition of an antigen by B lymphocytes triggers the production of antibodies. The T-cell receptors are only present on the cell surface of T lymphocytes, and they do not produce any soluble molecules similar to the immunoglobulins.

The antigen receptors of T and B lymphocytes belong to the immunoglobulin super gene family and are derived from a common ancestor. Immunoglobulins are composed of two identical heavy (H) chains and two identical light (L) chains. The T-cell receptor has an antigen-binding site consisting of either α and β chains or γ, δ, and ϵ chains. Circulating antibodies are structurally identical to B-cell antigen receptors but lack the transmembrane and intracytoplasmic sections.

1.6.1 T-Cell Receptor (TCR)

The T-cell receptor (TCR) is composed of integral membrane protein which recognizes the antigen, and as a consequence, its activation produces an immune response to eliminate the antigen. This process results in the development of $CD4^+$ or $CD8^+$ cells from the precursor T cells. The process involves other cell surface receptors and downstream signal transduction mechanisms.

The TCR is a member of the immunoglobulin super family and is composed of an N-terminal immunoglobulin variable domain, an immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail. Three hypervariable complementarity-determining regions are present in the variable domain of both TCR-α and TCR-β. Additional areas of hypervariability are present in the variable region of the β chain, which are not considered complementarity-determining regions due to their inability to contact the antigen. The processed antigen is recognized by complementarity-determining region-3, and additional interactions involve the N-terminal part of the antigen with complementarity-determining region-1 of the α chain and C-terminal part of the antigen with complementarity-determining region-1 of the β chain. The major histocompatibility complex is recognized by complementarity-determining region-2. The TCR falls into two groups, TCR-1 (γδ) and TCR-2 (αβ). More than 95 % of the TCR are TCR2. Both types of T cells arise from hematopoietic precursor cells.

1.6.1.1 γδ TCR (TCR-1)

The structure of γδ TCR is similar to αβ heterodimer. The generation of the γ chain involves VJ recombination, and the generation of δ chain requires V(D)J recombination. However, they differ from TCR-2 in terms of the types of antigens recognized, the mode of antigen presentation and recognition, and signal transduction pathways. Antigen recognition by γδ TCR is similar to antigen recognition by antibodies. They recognize intact protein antigens as well as nonprotein antigens. These TCRs exhibit a limited diversity, which may imply that they may have few natural ligands. Most of their known ligands are not of foreign origin; instead, they are of host origin. There is a correlation between Vγ and Vδ genes expressed by a γδ T cell and its function. This may be due to its function being determined by the interaction between particular TCR-V domains and function. Furthermore, complementarity-determining region-3 of the TCR-δ chain may be involved in determining the receptor specificity without any consideration for Vγ and/or Vδ.

1.6.1.2 αβ TCR (TCR-2)

TCR-2 is a heterodimer with a molecular weight of 90 kD and is made up of two peptide chains, an α chain (45 kD) and a β chain (40 kD). Each chain is composed of distinct constant and variable regions. These regions contain disulfide intrachain, which has partial homology with the immunoglobulin domains. The α chain is generated by VJ recombination and the β chain is generated by V(D)J recombination. Each of the V regions contains three hypervariable regions that make up the binding site. TCR-2 exhibits tremendous diversity, which is due to a unique combination of segments at the intersection of the specific regions, plus palindromic and random nucleotide additions. They recognize only membrane-bound processed antigens in the context of MHC molecules.

1.6.2 CD28

CD28 is expressed on T cells and is a part of costimulatory signal required for the activation and proliferation of T cells in response to an antigen. T-cell induction via CD28, in addition to the TCR, results in the synthesis and secretion of a number of cytokines, specifically IL-6. CD28 is the receptor for CD80 (B7.1) and CD86 (B7.2). The expression of CD86 on antigen-presenting cells is constitutive, whereas the expression of CD80 on antigen-presenting cells is augmented by Toll-like receptor ligands. Naïve T cells only constitutively express CD28, which is a B7 receptor. A lack of CD28 and B7 interaction during interaction of the TCR of a naïve T cell with antigen in the context of the MHC molecules culminates in the production of anergic T cells. The effective signaling by CD28 requires an intracellular domain with several residues, which it possesses.

1.6.3 CD40

CD40 is a costimulatory receptor present on the antigen-presenting cells. The expression of this molecule is diverse and is constitutively expressed on macrophages, dendritic cells, and B cells. Its expression can also be induced on smooth muscle cells, endothelial cells, epithelial cells, and fibroblasts. CD40 binds to CD154 (CD40L) on T cells. Their binding activates antigen-presenting cells and produces a number of downstream effector functions/effects. CD40 plays a role in a number of immune and inflammatory functions. They include isotope switching, development of memory B cells, and the formation of germinal center. The expression of CD40 is regulated by AKNA, which is an AT-hook transcription factor. The TNFR-associated proteins bind to CD40 and mediate signal transduction. These TNFR-associated proteins include TRAF1, TRAF2, and TRAF6. This interaction may play an early role in the pathogenesis of Alzheimer's disease, since interaction of CD40 with its ligand is required for the activation of amyloid-beta-induced microglia.

CD40 is a secondary signal for the activation of macrophages after it binds to CD154 on T cells. The primary signal is IFN-ϒ, which is secreted by TH1 cells. As a result of binding of CD40 to CD154, there is an increased expression of CD40 and TNF receptors, which produces additional activation of macrophages. This results in the release of free radical oxygen, nitric oxide, and potent antimicrobial substances resulting in the killing of microbes. Resting B cells can be activated after CD40L on T cells recognizes CD40 expressed on B cells. The activation of B cells through T cells will result in cell division, production of plasma cells, and isotype switching.

1.6.4 B7

B7 is located on antigen-presenting cells. After it binds to CD28 on T cells, a secondary costimulatory signal is produced for antigen recognition by T cells in the context of the MHC molecules. CD28 plays a role in autoregulation and intercellular association. B7 is also expressed on T cells. There are two types of B7 molecules: CD80 or B7.1 and CD86 or B7.2. CD80 is present on activated B cells and monocytes. CD86 is present on antigen-presenting cells, provides a costimulatory signal after binding to CD28 on T cells, and is a member of immunoglobulin superfamily. The costimulatory molecules are necessary to continue the immune response, and their interaction results in the activation and survival of T cells. There are additional signals, which play a role in the activation of the immune response. One example is the binding of 4-1BBL on the APC to 4-1BB (CD137) on T cell.

CD80/CD86 also binds to another molecule on T cells called CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and antagonizes the effects of the binding of CD28 to CD80/CD86. CTLA-4 belongs to immunoglobulin superfamily and is present on $CD4^+$ helper T cells. It is similar to CD28 and is also found on regulatory T cells and may have a role in their function. As a consequence, CTLA-4 (CD152) produces an inhibitory signal for immune response. CTLA-4 recruits a phosphatase to the T-cell receptor and as a result suppresses its signal. Others have suggested a different mechanism according to which it may capture and remove B7.1 and B7.2 from the cell surface of the antigen-presenting cells. This would make them unavailable for CD28. CTLA-4 may also have additional mechanisms of action including signaling through PI3-kinase (phosphatidylinositide 3-kinases) and/or affecting cell motility. The effects of CTLA-4 on cell motility seem to follow a “reverse-stop signaling model,” when observed in the lymph nodes. In this scenario, TCR-induced “stop signal” is reversed by CTLA-4. The antigen-presenting cells and T lymphocytes require this signal for a firm contact.

1.7 T-Cell Activation, TCR Complex, and Signal Transduction

Activation of $CD4^+$ T cells fits a “two-signal model” although multiple variations exist between different T-cell types. The interaction of the TCR and CD28 on the T cell with MHC complex and B7 family members on the antigen-presenting cells

provides stimulus for the induction of $CD4^+$ T cells. CD28 molecule is essential for this activation since its absence results in anergy. This is followed by downstream signal transduction emanating from the TCR and CD28. Binding of the TCR to the antigenic fragment presented in context with MHC molecules on the antigen-presenting cells provides the first signal. This signal is necessary to activate the antigen-specific TCR on a T cell. As described, antigen-presenting cells include macrophages, B cells, and dendritic cells. Usually, in case of a naïve response, dendritic cells are involved. MHC class I molecules present short peptides to $CD8^+$ cells, whereas longer peptides are presented by MHC class II molecules to $CD4^+$ cells. The receptors on the antigen are stimulated by some stimuli, resulting in costimulation, which serves as the second signal. These stimuli include antigens of the pathogens, necrotic bodies, and heat shock proteins. CD28 is a costimulatory molecule expressed constitutively on naïve T cells. CD80 and CD86 expressed on the antigen-presenting cells serve as costimulators for naïve T cells. In addition, the T cells acquire other receptors such as ICOS and OX40 after stimulation. The expression of ICOS and OX40 is CD28 dependent. T cell only responds to the antigen after the production of a second signal, and this mechanism has been put in place to avoid autoimmune responses.

The TCR is associated with CD3 (Fig. 1.13) resulting in the formation of TCR–CD3 membrane complex. The signal transduction is carried out by accessory molecules after the processed antigen comes in contact with T cell. CD3 is composed of five polypeptide chains, which together form three dimers. These include a heterodimer of γε, a heterodimer of δε, and either a homodimer of two ζ chains or a heterodimer of ζη chains. The γ, δ, and ε chains of CD3 belong to the immunoglobulin super family, as these chains possess an immunoglobulin-like extra domain, a transmembrane region, and a cytoplasmic domain. Copies of a sequence motif called immunoreceptor tyrosine-based activation motif (ITAMs) are present in the cytoplasmic tails of the γ, δ, ε, and ζ subunits. ITAMs serve as substrates for tyrosine kinase as well as binding sites for SH2 domains for other kinases. Members of both Src family (LcK) and Syk family (ZAP-70) of kinases play an important role in the recruitment of protein kinases to activate T-cell receptor. An ion pair is formed by the transmembrane domains of TCR-α and CD3δ, which is essential for the assembly and expression of the TCR. The transmission of signals from antigen-induced TCR to downstream transduction pathways also involves lateral association between transmembrane domain helices. CD45-associated proteins are involved in binding kinases with its intracellular domain and CD45 through transmembrane domain–transmembrane domain interaction. These proteins promote induction of kinases by the phosphatase CD45. The phosphorylation of intracellular tyrosines on T-cell receptor-interacting molecule (pp30) is crucial for the recruitment of phosphatidylinositide 3-kinases to the membrane. TCR–CD3 complex may be present not only in a monovalent but also as several different multivalent forms on uninduced resting T cells. These multivalent receptors play a critical role in recognizing low doses of antigens and are responsible for sensitivity. In contrast, a wide dynamic range is conferred by coexpressed monovalent TCR–CD3 complex.

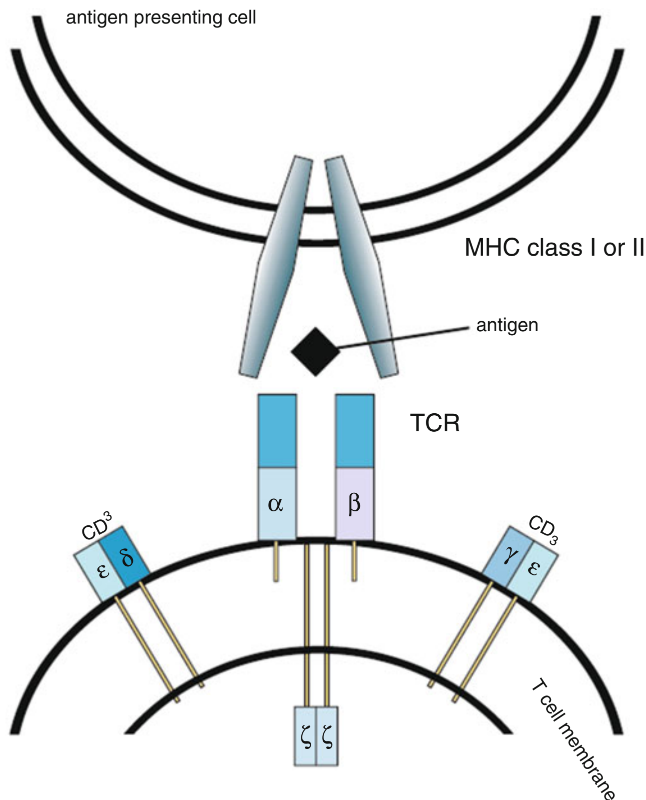

Fig. 1.13 The structure of T-cell receptor and antigen presentation. The T-cell receptor (TCR-2) is a heterodimer which is composed of two transmembrane glycoprotein chains α and β which are disulfide-linked polypeptides. TCR-1 is structurally similar but composed of γ and σ polypeptides. The T-cell receptor is also associated with the CD3 complex. CD3 complex is composed of six polypeptide glycoprotein chains known as CD3γ, CD3σ, CD3ε, and another protein known as ζ. When the T-cell receptor recognizes antigen, the CD3 complex is involved in signal transduction. The antigen is recognized by the TCR only in the context with MHC molecules after it is taken up and processed by the antigen-presenting cells

After activation of the TCR, there is induction of Src family tyrosine kinases ($p56^{lek}$), which phosphorylates phospholipase Cγ1. This is followed by the hydrolysis of phosphatidylinositol 4, 5 biphosphate, resulting in the production of diacylglycerol (DAG) and inositol triphosphate (IP_3). Protein kinase C is activated by DAG, which phosphorylates Ras. Ras is a GTPase and its phosphorylation induces Raf and initiation of MAP kinase signaling pathway. IP_3 is involved in calcium-dependent activation of IL-2 gene expression via nuclear factor of activated T cells (NFAT).

Another signal transduction mechanism involves phosphoinositides 3-kinases (PI3Ks), which phosphorylate phosphatidylinositol 4, 5 biphosphate, resulting in the activation of phosphatidylinositol triphosphate activating pathways. These pathways overlap with PLC gamma and are PI3K specific. PI3K-specific pathways

involve Akt-mediated inactivation of FOXO transcription factors. Furthermore, this pathway also includes glucose uptake and metabolism, which is transcription independent. P110delta also plays a role in this process, which has an isoform of PI3K and primary source of its activity. In addition to TCR and CD3, other molecules including CD4, CD8, LFA1, LFA2, CD28, and CD45R, termed as accessory molecules, play a role in antigen recognition and induction of T cells.

CD4 is present on helper T cells and CD8 is present on cytotoxic T cells. CD4 works as a monomer with four Ig domains in its extracellular protium, whereas CD8 functions as a dimer involving either an α and a β chain or two identical α chains. One extracellular IgV domain in their extracellular protium is present in CD8α and CD8 β molecules. CD8 αα and CD8 αβ are structurally similar but have notably different functions. The CD8 αα does not support conventional positive selection, and its expression does not require recognition by TCR in the context of MHC molecules. In contrast, CD αβ has a role as a co-receptor for the TCR on MHC class I-restricted mature T cells and thymocytes. The specific modulation of TCR activation signals to promote their survival and differentiation is mediated by CD8 αα. These CD αα receptors are expressed on agonist-triggered immature thymocytes, antigen-induced CD αβ T cells, and mucosal T cells. Antigenic stimulation through TCR results in the expression of CD8 αα. A number of other molecules play an accessory role as well. LFA-1, an adhesion molecule, strengthens the interaction of the T cell with the antigen-presenting cell. CD28, present on helper T cells, binds to B7 on antigen-presenting cells, which serves as a costimulatory signal, resulting in the induction of the T lymphocytes (Fig. 1.14). In the absence of CD28-B7-mediated signal, T lymphocytes do not respond, resulting in anergy.

The specificity of antigen recognition T-cell response is dependent on the interaction between the TCR, the MHC peptide complex, and the APC. The selection of the T-cell repertoire in the thymus and the induction of peripheral mature T cells seem to be dependent on the binding kinetics of the TCR and the MHC peptide complex. Particularly the half-life of the TCR–MHC interaction plays a significant role in T-cell activation. The significance of this kinetic binding parameter is observed in the capacity of T cells to respond to invading pathogens, autoimmunity, and tumor-produced antigens. They protect the body from reacting to the self. The MHC complex, which binds to the TCR with a short half-life, does not result in T-cell activation and in some cases may even have an inhibitory effect in response to a stimulus. In contrast, prolonged half-lives also disrupt T-cell induction, suggesting a set half-life is required for producing an optimal response. This restricted immune response serves as a deterrent against autoimmune diseases. The kinetics of the TCR–MHC interaction also determines the nature of the response by the peripheral mature T cells.

The regulation of T-cell responses during T-cell development and in mature T cells is dependent on the modulation of TCR expression. The rate constants for synthesis, endocytosis, recycling, and degradation are critical for TCR expression levels. There is a slow and constitutive cycling of TCR between the plasma membrane and the intracellular compartment in resting T cells. The di-leucine-based (diL) receptor-sorting motif in the TCR subunit CD3γ is required for constitutive

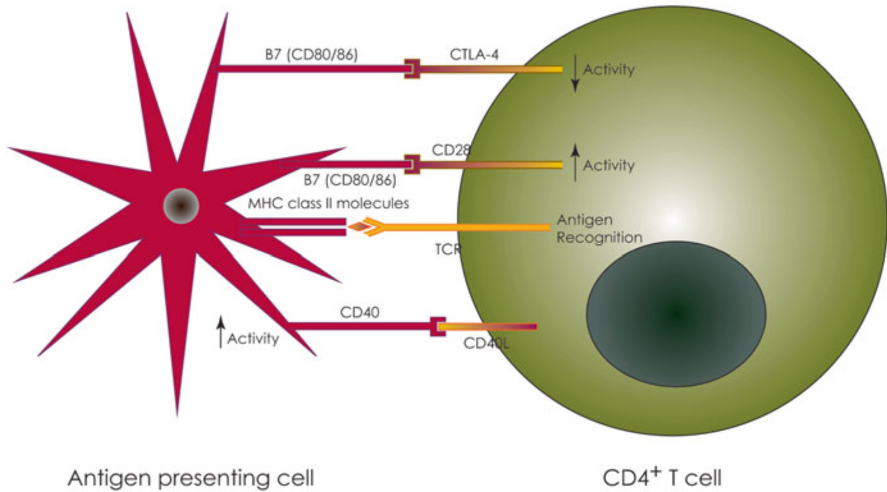

Fig. 1.14 Costimulatory molecules in antigen recognition: Two signals are required for T cells to become completely activated. The first signal results from the recognition of processed antigen and its presentation to TCR in the context of MHC molecules. The second signal while antigen non-specific involved the interaction of CD28 molecules on T cells with CD80/CD86 (B7.1/B7.2) on the antigen-presenting cells is termed costimulatory signal. This costimulation is required for the proliferation, differentiation, and survival of T cells. A natural ligand CTLA-2 present on the T cells blocks this interaction and provides a negative feedback mechanism. CD40 is also a costimulatory molecule present on antigen-presenting cells and is responsible for their activation

TCR cycling. The quality control of the TCR may be regulated by this event. TCR downregulation is dependent on an enhancement in endocytic rate constant induced by the TCR. The endocytosis of triggered TCR results either from ubiquitination of the TCR as protein tyrosine kinase is activated or PKC-dependent induction of the diL motif. Furthermore, PKC/CD3g-dependent pathways are responsible for the endocytosis of non-triggered TCR. The signaling is inhibited by TCR downregulation. Alternatively, internal stores of TCR can be used by the immune system to recognize antigens.

1.8 Pre-TCR

CD4 and CD8 receptors are not present on early T-cell progenitors in the thymus. These cells express a nascent TCR-β chain and an invariant pre-TCR-α chain and CD3 receptors. This is followed by the generation of a functional T-cell receptor β chain on these pre-TCR-expressing thymocytes. A process of β chain selection induces the expression of CD4 and CD8 antigens on these thymocytes and their differentiation into distinct subsets. The T-cell development utilizes the pre-TCR as a

key molecular sensor. The rearrangement of the TCR-α and $CD4^+$ and $CD8^+$ cell survival and proliferation is induced by the pre-TCR. Other signaling molecules including CD3ε, Syk, Fyn, Lck, and Zap-70 participate in pre-TCR-mediated differentiation and signaling. Pre-TCR-β-mediated selection is further supported by IL-7 and Notch ligand and may require an exogenous ligand on thymic stroma. However, most of the data suggests that this may not be the case and this signaling may be stimulus independent. Pre-TCR is involved in the immune response in an autonomous and ligand-independent manner. The autonomous signaling is a function of $CD4^-CD8^-$ thymocytes where the pre-TCR signaling takes place.

1.9 Antigen Recognition

The antigens are recognized by the immune system by utilizing antibodies generated by B cells and by TCR on the T cells. However, the mechanisms by which T cells and B cells recognize antigen are different. Both the antibodies and T cells are capable of recognizing a wide range of antigens. The antibodies and the TCR have many similar features. They both possess variable (V) and constant (C) regions, and the process of gene recombination, which produces the variable domains from V, D, and J gene segments, is also similar for each type of receptor. However, T cells and antibodies recognize the antigens differently: antibody recognizes antigens in solution or on cell surfaces in their native forms, while T-cell receptors do not recognize antigen in their native forms. The antigens are recognized by T cells only after they are processed and then only in context with MHC molecules. Antigens recognized by T cells are first taken up by the macrophages or other antigen-presenting cells and processed so that the determinant recognized by the T-cell antigen receptor is only a small fragment of the original antigen.

A second difference between the antibody and the TCR is that the antibody can be produced in two forms, secreted or membrane bound, whereas the TCR is always membrane bound. The secreted antibody has a dual function, where the V domains bind antigens and the C domain binds to the Fc receptors.

1.10 Antigen Presentation

The fragments of intracellular components of cells, infected with an invading pathogen, in the context of MHC molecules inform the T cells about their presence. Alternatively, many invading organisms due to their large size cannot be recognized by the T cells and need to be processed and presented by antigen-presenting cells. To recognize and eliminate intracellular antigens, cytolytic T cells are involved in a molecular health screening program to protect nucleated cells from infection and disease including cancer. To protect the cell, the host cells digest its own cytoplasmic proteins by proteasome into small fragments, followed by their transportation using the transporter associated with antigen processing (TAP) complex, into the

endoplasmic reticulum. The antigenic peptide fragments then associate themselves with MHC molecules and migrate to the cell surface for checking by cytolytic T cells periodically. The cytolytic cells recognize antigens in the context of MHC class I molecules, causing the cell death.

For larger or extracellular antigens, there is phagocytosis of the invading pathogen by the dendritic cells followed by their migration to T-cell-enriched lymph nodes. This migration is facilitated by chemotactic signals, during which a maturation phase allows the dendritic cells to lose their ability of phagocytosis. Furthermore, the dendritic cells acquire ability of enhanced communication with T cells in the lymph nodes. Other pathogen-associated molecular pattern molecules (PAMP) provide signals for this maturation of the dendritic cells via pattern recognition receptors (PRRs). PRRs are proteins present on the cells of the innate immune system and are a primitive part of immunity. They are classified according to their ligand specificity.

In the dendritic cells, invading organism-associated proteins are digested by lysosome-associated enzymes. The digestion produces small peptides, which in the lymph node are expressed by the dendritic cells on their cell surface coupled with the MHC molecules. The T cells migrating through the lymph nodes recognize the antigen on the surface of the dendritic cells in the context of the MHC molecules. The extracellular antigens are generally presented to helper T cells in the context of MHC class II molecules, which are expressed in large numbers on dendritic cells.

1.11 Antigen–Antibody Binding

The binding of antigen to antibody involves the formation of multiple non-covalent bonds between the antigen and amino acids of the antibody. Antigen–antibody complex results from hydrogen bonding, electrostatic bonding, van der Waals bonds, and hydrophobic bonds. A considerable binding energy is produced as a result of multiple types of bonds.

1.12 The Structure of Antigens

The antigens are three-dimensional structures and present many different configurations to B cells, resulting in a high number of different possible antibodies. Furthermore, different antibodies to an antigen often bind to overlapping epitopes. This allows the binding of different antibodies to a particular antigenic region of the molecule, without binding exactly to the same epitope.

Technically, antibodies may be produced against any part of the antigen, but this is usually not the case. Certain areas of the antigen are particularly antigenic and that the majorities of the antibodies bind to these regions that are called immunodominant regions. These regions are present at exposed areas on the outside of the antigen, where there are loops of polypeptide lacking a rigid tertiary structure and could be very mobile.

1.13 T-Cell Antigen Recognition

As already stated, T cells do not see unprocessed antigens. Another requirement for antigen recognition by T cells is that they recognize antigen on the surface of other cells, called the antigen-presenting cells. These cells could also be virally infected cells, which then can be killed by cytotoxic T cells. The antigen is taken up by the APC and is degraded, so only a small fragment of the antigen can be presented to the TCR in the context of MHC molecules. Interference in the internal processing of the antigen in the APC will result in their inability to present the antigen.

The interaction between the T cells and the targets is said to be genetically restricted. For example, cytotoxic T cells specific for a particular virus will only recognize it in the infected cells of their own MHC haplotype. This is also the case for the recognition of helper T cells. Consequently, the T cell recognizes both the antigen and the MHC molecules on other cells.

The processed antigen must be physically associated with the MHC molecules, which interacts with MHC molecules and the TCR via amino acid residues.

1.14 Major Histocompatibility Complex

The rejection of grafted tissues provided clues about the existence of a very diverse major histocompatibility complex (MHC). Immune system must discriminate "nonself" from "self," which results in the rejection of the grafted tissue and response to invading pathogens. This function is achieved via the molecules of the major histocompatibility complex (MHC). The MHC is based in multiple loci, which determine the acceptability of the grafted tissue. It also plays a crucial role in the development of the immune response since T cells recognize antigens only in context with the MHC molecules. The MHC complex is present on chromosome 17 in mice and on chromosome 6 in humans where it is called human leukocyte antigens (HLA). Highly polymorphic loci constitute the MHC complex and are closely linked. In humans, MHC region on chromosome 6 is about 3.6 Mb, which contains 140 genes between flanking genetic markers MOG and COL11A2. Half of them have known immunologic functions. Every antigen, both nonself and self, is recognized by T cells in context with MHC molecules. $CD4^{+}$ T cells recognize antigens in the context of class II MHC molecules, whereas CD8 T cells recognize antigens in the context of class I MHC molecules. During embryogenesis, T cells recognizing self-antigens in association with MHC molecules are eliminated, whereas T cells recognizing foreign antigens in the presence of MHC molecules are retained. Breakdown in this process of self-recognition and elimination can cause autoimmune diseases. In contrast, failure to recognize foreign antigens may result in an immunodeficient state, resulting in infections and the development of tumors.

1.14.1 Major Histocompatibility Complex Molecules

The MHC region is divided into three subgroups, called MHC class I molecules, MHC class II molecules, and MHC class III molecules. MHC class I and class II molecules are membrane-bound glycoproteins that are similar in structure and function. The peptide conformations of MHC class I and MHC class III molecules are shown in Fig. 1.15. However, the class I and class II molecules are distinguishable on the basis of their structure, tissue distribution, and function. Class I molecules include the HLA-A, HLA-B, and HLA-C. They are present on all nucleated cells and encode peptide-binding proteins and antigen-processing molecules including Tapasin and Tap. Class II molecules include HLA-DR, HLA-DQ, and HLA-DP. They are located on antigen-presenting cells and encode heterodimeric peptide-binding proteins and proteins, which regulate adding of peptides to MHC class II molecule proteins in the lysosomal compartment. Class III molecules include complement system. These are soluble molecules and do not act as transplantation antigens, nor do they present antigen to T cells. A comparison of the characteristics of MHC class I and MHC class II molecules is shown in Table 1.8.

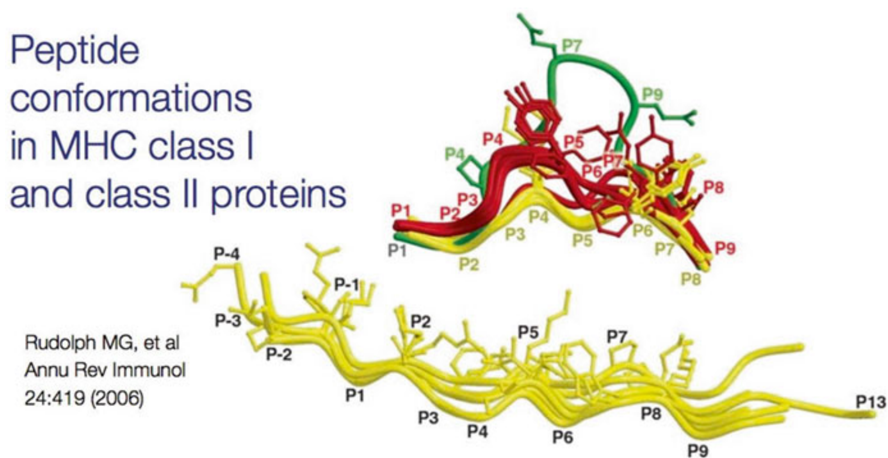

Fig. 1.15 Structure of MHC molecules (Source: Rudolph et al. (2006) How TCRs bind MHCs, peptides and coreceptors. *Annual Review of Immunology*. 24:419–466. Annual Reviews. Reproduced with Permission)

Table 1.8 Comparison of class I and II HLA molecules

Properties	Class I	Class II
Antigen	HLA-A, HLA-B, HLA-C	HLA-D, HLA-DR, HLA-DQ, HLA-DP
Tissue distribution	All nucleated cells	Antigen-presenting cells, B cells
Function	Present processed antigenic fragments to CD8 T cells. Restrict cell-mediated cytolysis of virus-infected cells	Present processed antigenic fragment to CD4 cells. Necessary for effective interaction among immunocompetent cells

1.14.1.1 Structure of Class I HLA Molecules

MHC class I molecules are heterodimers and made up of an α chain and β_2 microglobulin. The α chain is composed of three polymorphic domains, α_1, α_2, and α_3, with a peptide binding groove between α_1 and α_2 chains. The peptides derived from cytosolic proteins bind to this groove. Eight β-pleated sheets and two α helices form this groove, and both chains are non-covalently linked. The α chain contains 338 amino acid residues. Class I HLA molecules are present on all nucleated cells. For an antigen to be recognized by a CD8 T lymphocyte, the antigen must be recognized in combination with a class I molecule. Figure 1.15 depicts the structure of MHC class I and II molecules.

1.14.1.2 Class II Molecules

MHC class II molecules are made up of two membrane-spanning proteins; each chain has a size of 30 kD and is made up of two globular domains. These domains are called α-1, α-2, β-1, and β-2. Each chain possesses an immunoglobulin-like region next to the cell membrane. MHC class II molecules are highly polymorphic.

Class II HLA molecules have a limited cellular distribution as they are found on APCs, which include B lymphocytes, macrophages, and dendritic cells. In addition, some cells that do not normally express class II molecules (such as resting T cells, endothelial cells, thyroid cells, as well as others) can be induced to express them. The function of class II molecules is to present processed antigenic peptide fragments to CD4 T lymphocyte during the initiation of immune responses. Just as CD8 T lymphocytes recognize peptide fragments only in the context of a class I molecules, CD4 T lymphocytes recognize peptide fragments only in the context of class II molecules. The HLA-A, HLA-B, and HLA-C genetic loci determine the class I molecules that bear the class I antigens, and the HLA-DR, HLA-DQ, and HLA-DP genetic subregions, each of which contains several additional loci, determine the class II molecules that bear the class II antigens.

1.15 Cellular Migration

1.15.1 Cell-Adhesion Molecules

The migration of molecules generated in blood and leukocytes into the tissues is controlled by the vascular endothelium. The passage through the endothelial cells, lining the walls of blood vessels, is required for leukocytes so they can enter an area of injury, inflammation, or into the peripheral lymphoid organs. This process is called extravasation. Leukocyte-specific cell-adhesion molecules are expressed on endothelial cells. Their expression could be constitutive, while others are expressed in response to cytokines during an inflammatory response. Many different types of

circulating leukocytes including lymphocytes, monocytes, and neutrophils express cell surface receptors, which recognize and bind to cell-adhesion molecules on vascular endothelium. There are many types of adhesion receptors on the cell surface of which four major families include most of these receptor types. They are classified as integrins, selectins, immunoglobulin super family, and cadherins. In addition to cellular migration, they are involved in a number of important cellular functions including growth, differentiation, mutagenesis, and cancer metastasis. They also transmit information into the cell from the cellular matrix (Fig. 1.16).

1.15.2 Integrins

Integrins are non-covalently linked heterodimeric membrane proteins with two subunits, an α and a β chain. These transmembrane proteins are constitutively expressed, but require activation for binding to their ligands. After activation, a signal is transmitted from the cytoplasm resulting in a modification in the extracellular domains of integrins. This modification in the conformation of these domains results in an increased affinity of the integrins for their ligands. When bacterial peptides stimulate leukocytes, "inside out" signaling occurs, resulting in an increase in the affinity

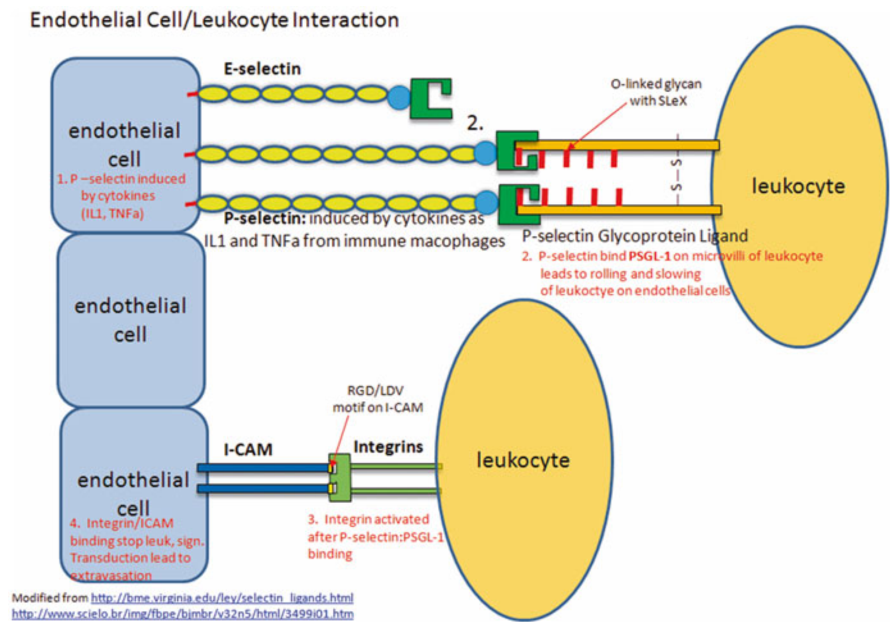

Fig. 1.16 This figure depicts the interaction of leukocytes and endothelial cells via adhesion molecules. The adhesion molecules include integrins, selectins, and ICAMs (Source: BioWiki.ucDavis.edu)

of the leukocyte integrins for members of the immunoglobulin family. The binding of an integrin to its ligand results in "outside in" signaling, which may regulate cell proliferation and apoptosis.

Integrins are expressed on leukocytes, and different subsets of leukocytes express different integrins. Fifteen different α and eight different β units have been identified, which can combine in various ways to express different integrin receptors. Integrins have three activation states, basal avidity, low avidity, and high avidity. They play a pivotal role in immunity, cancer, homeostasis, and wound healing, which is mediated via transmitting signals bidirectionally across the plasma membrane. The affinity to the ligand is regulated by global conformational changes, which relate to the inter-domain and intra-domain shape shifting. The downward movements of the C-terminal helices play a critical role in integrin conformational signaling.

1.15.3 Selectins

Selectins are a family of membrane glycoproteins that are divalent cation dependent and bind to specific carbohydrates containing sialylated moieties. They are composed of leukocyte (L)-selectins, endothelial (E)-selectins, and platelet (P)-selectins. L-selectins are expressed on most leukocytes, whereas vascular endothelial cells express E-selectins and P-selectins. Initial binding of leukocytes with vascular endothelium involves selectins, and consequently they play an important role in leukocyte trafficking.

Circulating leukocytes bind to the endothelium via selectins expressed on vascular endothelium or leukocytes. Selectins have a low binding affinity but causes the leukocytes to slow down as they roll on endothelial cells. During this slowdown period, chemoattractants activate leukocytes. This activation increases the affinity of their integrin (β2) receptors for ligands on activated endothelial cells. The leukocytes migrate between endothelial cells of venule to the site of injury or inflammation as a result of a chemotactic signal emanating outside the venule. The functions of selectins were identified by using knockout mice for each gene. L-selectin-deficient mice have an impaired homing of lymphocytes to lymph nodes. In P-selectin-deficient mice, leukocytes roll at sites of inflammation, but do not roll along normal blood vessels. E-selectin-deficient mice are normal, but both P and E-selectin-deficient mice leukocytes do not roll even at sites of inflammation.

1.15.4 Immunoglobulin Superfamily

These are calcium-independent transmembrane glycoproteins that contain a variable number of immunoglobulin-like domains. This group includes intercellular adhesion molecules (ICAM), vascular cell-adhesion molecules (VCAM), neural cell-adhesion molecules (NCAM), and platelet-endothelial cell-adhesion molecules

(PECAM). ICAM and VCAM are present on vascular endothelial cells and bind to various integrin molecules or other immunoglobulin superfamily cell-adhesion molecules. The NCAM-1 found predominantly in the nervous system is involved in neuronal patterns and mediates homophilic interaction. By associating laterally with fibroblast growth factor receptor, NCAM stimulates tyrosine kinase activity associated with that receptor, resulting in the induction of outgrowth of neurites. PECAM-1 plays a role in inflammation and immune response.

1.15.5 Cadherins

Cadherins are calcium dependent and establish molecular links between adjacent cells. They include neural cadherins (NC), epithelial cadherins (EC), placental cadherins (PC), protocadherins, and desmosomal cadherins. Cadherins mediate homophilic interactions and form zipper-like structures at adherence junctions and are linked to cytoskeleton through the catenins. The ability of interaction of catenins with the intracellular domain confers their adhesive properties. Cadherins play a crucial role in embryonic development and tissue organization.

All adhesion molecules after binding to their ligands and/or due to clustering of their receptors as a result of interaction with the ligand transduce signals across the membrane. Interactions with downstream signaling and cytoskeleton take place after conformational changes in the receptors, which are followed by the reorganization of the cytoskeleton. There is phosphorylation and dephosphorylation of a number of molecules as a result of a signaling cascade initiating from the receptor activation that causes conformational changes in a number of cytoplasmic kinases. The signal produces the synthesis of new proteins such as cytokines, soluble adhesion molecules, and metalloproteases as a result of induction in gene expression. Defective interactions between adhesion molecules cause disease.

1.16 Immune Tolerance

Immune tolerance is defined as inability of the immune system to be nonresponsive to nonself, for which it is capable to produce an immune response. It is opposite of regular destruction of an antigen or foreign substance or tissue via production of humoral or cellular response. There are two types of tolerance: central and peripheral tolerance. The central tolerance originates in the primary lymphoid organs, bone marrow, or thymus, whereas the peripheral tolerance originates in the secondary lymphoid organs such as lymph nodes, as well as other tissues. The mechanisms of the central and peripheral tolerance are different, but there is no difference in the outcome.

The immune system is capable of differentiating self from nonself via predominantly central tolerance. Peripheral tolerance provides a negative feedback for the

immune response, so it will not go unchecked beyond its need. A lack of normal immune tolerance results in a number of diseases, including autoimmune disorders such as rheumatoid arthritis, scleroderma, Sjogren's syndrome, systemic lupus erythematosus, ankylosing spondylitis, and type 1 diabetes. Other diseases may include inflammatory bowel disease, allergy, and asthma.

Tolerance could also produce some undesired effects, such as the ability of some microbes to avoid detection, infect the host, and cause the disease in the absence of a generation of immune response against them. Furthermore, the induction of peripheral tolerance in the local microenvironment helps a number of different types of cancers to avoid a robust immune response resulting in their destruction.

1.16.1 Central Tolerance

Central tolerance involves the deletion of the precursors of the autoreactive lymphocytes before their differentiation and maturation. For B cells it happens in the bone marrow and for T cells in the thymus. The bone marrow and the thymus are primary lymphoid organs, where bone marrow cells, medullary thymic epithelial cells, or thymic dendritic cells present lymphocytes undergoing maturation to self-antigens. The presence of self-antigens is attributed to their endogenous expression and migration through circulating blood from the peripheral tissue. The transcription factor, autoimmune regulators (AIRE), is expressed in the medulla and the thymus and is responsible for controlling the immune response from attacking the self. AIRE is involved in the expression of proteins of other nonthymic tissues in thymic stromal cells. Self-recognizing antigens are eliminated as a result of apoptosis or the development of anergy. A lack of response by defense mechanisms to invading pathogens due to induction of peripheral lymphocyte tolerance is defined as anergy. For B-cell central tolerance, immature B cells that only express cell surface IgM molecule undergo negative selection. This is achieved by binding to self-molecules, which are present in the bone marrow. B cells encounter multivalent cell surface or low valence soluble antigens. The clonal deletion is via apoptosis. Before apoptosis, an interval allows the rescue of self-reactive B cells by gene rearrangements. This may cause the replacement of the self-reactive receptor with a non-autoreactive receptor. The soluble self-antigens recognizing immature B cells are not deleted; however, they are unable to express IgM on their cell surface.

Some autoreactive T cells may not get eliminated and also do not respond to the stimulus of their B-cell receptor. This is termed as state of immunologic ignorance attributed to weakly autoreactive B cells. The same situation also exists for T cells, where natural regulatory T cells are the product of the differentiation of weakly self-recognizing T cells. The natural regulatory T cells may play a role in inhibiting peripheral T-cell autoreactivity. This leads to their migration to the periphery, while only expressing IgD without the ability to respond to the antigens. The B cells that recognize foreign antigen in the periphery express both IgM and IgD receptors and do not come in contact with the antigen, as they are mature in the bone marrow.

The elimination of autoreactive T cells is more necessary than autoreactive B cells. The ability of B cells to recognize a wide array of antigen is preferable, which will result in the production of antibodies to a great variety of invading pathogens. The autoreactivity is controlled due to the fact that the full activation of B cells requires confirmation by self-restricted T cells. It is necessary that these self-restricted T cells recognize the same antigen. In contrast, autoreactive T cells that are not eliminated have a potential to cause self-damage, which reiterates a strict need for their destruction. The selection of T cells is much more rigorous as they undergo both positive and negative selection to produce T cells, which will recognize antigens in the context of the MHC molecules. T-cell tolerance takes place in the thymus. The positive selection takes place in the thymic cortex and is primarily mediated by thymic epithelial cells. The binding of a maturing T cell to the surface of MHC molecule saves it from apoptosis. The cells which are unable to recognize MHC molecules on thymic epithelial cells will face destruction. As a consequence, the cells capable of recognizing antigens in association with MHC molecules are retained. This allows T cells to recognize infected host. The differentiation of a T cell into a $CD4^+$ or $CD8^+$ T cells is also determined by positive selection. The thymocytes carry both $CD4^+$ and $CD8^+$ receptors. Depending on their recognition of either MHC class I or MHC class II, they are transformed to $CD8^+CD4^-$ and $CD4^+CD8^-$ cells, respectively.

Some T cells also go through negative selection that takes place in the cortex, at the corticomedullary junction, and the medulla. In the medulla, it is mediated by dendritic cells or medullary thymic epithelial cells (mTECs). The developing T cells recognize mTEC and generate signal for the apoptosis of self-reactive T cells. As a result, autorecognizing T cells are deleted. This pathway is regulated by AIRE and is dependent on the tissue expression of tissue-specific antigens (TSAs). All possible self-reactive T cells cannot be deleted by clonal deletion of T cells in the thymus. Deletion in the periphery is required for T cells recognizing antigens, which are not present in the thymus or are expressed after puberty. The process of negative selection is essential to protect from autoimmune diseases. This process is most active during fetal development, but continues throughout life, and slows as the thymus and the bone marrow regress. T and B cells that are autoreactive are eliminated by negative selection, whereas the cells recognizing the nonself/antigens derived from pathogens are retained.

1.16.2 Peripheral Tolerance

The development of peripheral tolerance takes place after the maturation of B and T cells. This occurs after they migrate to the lymph nodes and peripheral tissues. It mostly involves the $CD4^+$ helper T cells, which drives the entire immune response including the antibody production by B cells. In some cases, there may be undesired auto-recognition due to a T cell, which was not eliminated or may express some receptors that recognize self. These autoreactive T cells that were not deleted during

negative selection in the thymus need to be destroyed in the periphery. In the periphery and/or lymphoid tissue, there is development of regulatory T cells (iTreg cells), which are derived from naïve CD4^{+} helper T cells. This process is driven by IL-2 secreted by T cells and TGF-β secreted by tolerizing dendritic cells, other antigen-presenting cells, or even the surrounding tissue in some cases. In addition to regulatory T cells, other cell types are also involved in this process. They include TR1 cells (IL-10 secreting but not expressing Foxp3), TH3 cells (TGF-β secreting), and other less characterized cells. IL-10 and TGF-β are produced by a subset of B cells as well. CD22 is also expressed by B cells, which is a nonspecific inhibitor receptor, and it attenuates B-cell receptor induction. Dendritic cells can cause anergy in antigen-recognizing T cells, in addition to synthesizing indoleamine 2,3-dioxygenase (IDO), which depletes tryptophan. Only some dendritic cells produce IDO, and T cells require tryptophan for proliferation. A decreased production of tryptophan will result in decreased ability of T cells to respond to an antigen by proliferation. Furthermore, immune-privileged tissues such as the anterior chamber of the eye, brain, and testis express Fasl (Fas ligand, CD95L), which is a member of the tumor necrosis factor family. Fasl can produce a programmed cell death caused by interaction of Fas ligand and Fas receptors, termed activation-induced cell death (AICD). AICD negatively regulates activated T cells that is a product of continuous induction of the T-cell receptors. This helps maintain immune tolerance and an inhibition of this mechanism may cause autoimmune disease. Both activated T cells and B cells express Fasl, and the cells expressing Fas receptors undergo clonal deletion by AICD. In case of activated T cells, they may kill each other or be killed by themselves.

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Chapter 2
Role of Cytokines

Abstract This chapter described the physiologic, pharmacologic, and pathologic effects of cytokines. They are glycosylated small polypeptides, synthesized and released by a variety of sources including lymphocytes, leukocytes, and other cell sources. Cytokines are a crucial component of immune response, as they aid in the intercellular communication and are involved in both health and disease, through their physiological and pathological effects. They are involved in mediating natural immunity and regulating the activation, growth, and differentiation of lymphocytes, leukocytes, as well as other cell types. Furthermore, they play a role in the regulation of immune-mediated inflammation and stimulate hematopoiesis. Their effects are mediated via cell surface receptors. Based on their physiological and pathological effects, they are ideal therapeutic agents, either by themselves or as antagonists, which also include specific monoclonal antibodies directed against them.

Keywords Interleukin (IL)-1 • Kineret • IL-2 • IL-37 • IL-2 • IL-2R • CD25 • Proleukin • LAK therapy • Denileukin diftitox • IL-15 • IL-4 • Airway hyperresponsiveness • Isotype switching • IL-5 • IL-6 • IL-9 • IL-10 • IL-22 • TH22 • TH17 • RORϒt • MAPK • CRF2-4 • Muc1 • Muc3 • Muc10 • Muc13 • β-defensin-3 • RegIIIβ • RegIIIϒ • IL-11 • Oprelvekin • IL-12 • IL-35 • IL-13 • IL-17 • IL-18 • IL-23 • Interferon-α • Roferon A • Peginterferon α -2a • Interferon-β • Interferon-γ • IL-28 • IL-29 • G-CSF • Filgrastim • Sargramostim • TNF-α • IL-7 • TNF-R1 • TNF-R2 • Etanercept • IL-32 • CXC chemokines • CC chemokines • C chemokines • CX3C chemokines • Autoimmune disease • Allergic disease • Inflammatory response • Acute phase response • Hepatitis B • Hepatitis C • Apoptosis • Sepsis • AIDS • Diabetes • Cancer • Rheumatoid arthritis • Asthma • Vascular endothelial cells • Chemokines • Chemokine receptors

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2.1 Introduction

Cytokines are small glycoproteins that are produced by a number of cell types, but predominantly by leukocytes, and regulate immunity, inflammation, and hematopoiesis. They regulate a number of physiologic and pathologic functions including innate immunity, acquired immunity, and a plethora of inflammatory responses. The discovery of cytokines was initiated in the 1950s, but the precise identification of their structure and function took many, many years. Original discoveries were those of interleukin (IL)-I, interferon (IFN), and nerve growth factors; however, they were purified and given the names many years later. Elucidation of the precise physiologic, pathologic, and pharmacologic effects of many of the cytokines is still in progress. The modern techniques in molecular biology were principally responsible for their complete identification and as a consequence, several hundred cytokine proteins and genes have been identified, and the process still continues.

Cytokines are produced from various sources during the effector phases of natural and acquired immune responses and regulate immune and inflammatory responses. They are also secreted during nonimmune events and play a role unrelated to the immune response in many tissues. Generally, their secretion is a brief, self-limited event. They are not only produced by multiple diverse cell types, but also they act upon many different cell types and tissues. Cytokines often have multiple effects on the same target cell and may induce or inhibit the synthesis and effects of other cytokines. After binding to specific receptors on the cell surface of the target cells, cytokines produce their specific effects. Multiple signals regulate the expression of cytokine receptors. The target cells respond to cytokines by new mRNA and protein synthesis, which results in a specific biological response.

2.2 Interleukin-1

Interleukin-1 was originally discovered as a factor that induced fever, caused damage to joints, and regulated bone marrow cells as well as lymphocytes; it was given several different names by various investigators. Later, the presence of two distinct proteins, IL-1α and IL-1β, was confirmed, which belong to a family of cytokines, the interleukin-1 superfamily. Ten ligands of IL-1 have been identified, termed IL-1F1-IL-1F10. With the exception of IL-IF4, all of their genes map to the region of chromosome 2. IL-1 plays an important role in both innate and adaptive immunity and is a crucial mediator of the host inflammatory response in natural immunity. The major cell source of IL-1 is the activated mononuclear phagocyte. Other sources include dendritic cells, epithelial cells, endothelial cells, B cells, astrocytes, fibroblasts, and large granular lymphocytes (LGL). Endotoxins, macrophage-derived cytokines such as TNF or IL-1 itself, and contact with $CD4^{+}$ cells trigger IL-1 production. IL-1 can be found in circulation following Gram-negative bacterial sepsis. It produces the acute phase response as a result of infection. IL-1 induces fever after bacterial and viral infections. It suppresses the appetite and induces

muscle proteolysis, which may cause severe muscle "wasting" in patients with chronic infection. Interleukin-1 β causes the destruction of β cells leading to type 1 diabetes mellitus. It inhibits the function and promotes the apoptosis of pancreatic β cells. Activation of helper T cells, resulting in IL-2 secretion, and B-cell activation is mediated by IL-1. It is a stimulator of fibroblast proliferation, which causes wound healing. Autoimmune diseases exhibit increased IL-1 concentrations. It suppresses further IL-1 production via an increase in the synthesis of PGE2.

IL-1s exert their effects via specific cell surface receptors that include a family of about nine members characterized as IL-1R1 to IL-1R9. All family members with the exception of IL-1R2 have an intracellular Toll-like receptor domain. Each type of receptor in the family has some common and some unique features. The ligands for these receptors are shown in Table 2.1.

2.2.1 Kineret (Anakinra)

Kineret is a human IL-1 receptor antagonist and is produced by recombinant DNA technology. It is non-glycosylated and is made up of 153 amino acids. With the exception of an additional methionine residue, it is similar to native human IL-1Ra. Human IL-1Ra is a naturally occurring IL-1 receptor antagonist and a 17-kDa protein, which competes with IL-1 for receptor binding and blocks IL-1's activity.

Kineret is recommended for the treatment of severely active rheumatoid arthritis for patients 18 years of age or older. It is indicated for patients who have not responded well previously to the disease-modifying antirheumatic drugs. It reduces inflammation, decreases bone and cartilage damage, and attacks active rheumatoid arthritis. The drug can be used alone or in combination with other antirheumatic drugs. However, it is not administered in combination with TNF-α antagonists. Kineret also improves glycemia and beta-cell secretory function in type 2 diabetes mellitus. It is administered daily at a dose of 100 mg/day by subcutaneous injection.

The most serious side effects of Kineret are infections and neutropenia. The injection site reactions are also common. Other side effects may include headache,

Table 2.1 IL-1 ligands and their receptors

Ligand name	Receptor name
IL-1F1	IL-1R1, IL-1R2
IL-1F2	IL-1R1, IL-1R2
IL-1F3	IL-1R1, IL-1R2
IL-1F4	IL-1R5
IL-1F5	IL-1R6
IL-1F6	Unknown
IL-1F7	IL-1R5
IL-1F8	Unknown
IL-1F9	IL-1R6
IL-1F10	IL-1R1

nausea, diarrhea, flu-like symptoms, and abdominal pain. The increased risk of malignancies has also been observed.

2.3 Interleukin-37

IL-37 is a member of IL-1 cytokine family and binds to IL-18 receptors (IL18R1/IL-1Rrp). It also binds to IL-18 binding protein (IL18BP). Since IL18BP is an inhibitory binding protein of IL-18, IL-37 inhibits the activity of IL-18.

2.4 Interleukin-2

IL-2, a single polypeptide chain of 133 amino acid residues, is produced by immune regulatory cells, which are principally T cells. When a helper T cell binds to an antigen-presenting cell using CD28 and B7, $CD4^+$ cells produce IL-2. IL-2 supports the proliferation and differentiation of any cell that has high-affinity IL-2 receptors. It is necessary for the activation of T cells. Resting T lymphocytes (unstimulated) belonging to either the $CD4^+$ or $CD8^+$ subsets possess few high-affinity IL-2 receptors, but following stimulation with specific antigen, there is a substantial increase in their numbers. The binding of IL-2 with its receptors on T cells induces their proliferation and differentiation.

IL-2 is the major growth factor for T lymphocytes, and the binding of IL-2 to its specific receptors on TH cells stimulates the proliferation of these cells and the release of a number of cytokines from these cells. IL-2 is required for the generation of $CD8^+$ cytolytic T cells, which are important in antiviral responses. It increases the effector function of natural killer cells. When peripheral blood lymphocytes are treated with IL-2 for 48–72 h, lymphokine-activated killer cells (LAK) are generated, which can kill a much wider range of targets including the tumor cells. IL-2 enhances the ability of the immune system to kill tumor cells and may also interfere with the blood flow to the tumors. It not only induces lymphoid growth but it also maintains peripheral tolerance by the generation of regulatory T cells. IL-2 knockout mice produce a wide range of autoantibodies and many die of autoimmune hemolytic anemia, which suggests it plays a role in immune tolerance.

2.4.1 IL-2 Receptors

The IL-2 receptor occurs in three forms with different affinities for IL-2; the three distinct subunits are the α, β, and γ chains (Fig. 2.1). The monomeric IL-2Rα possesses the low affinity, the dimeric IL-2Rβγ has intermediate affinity, and the

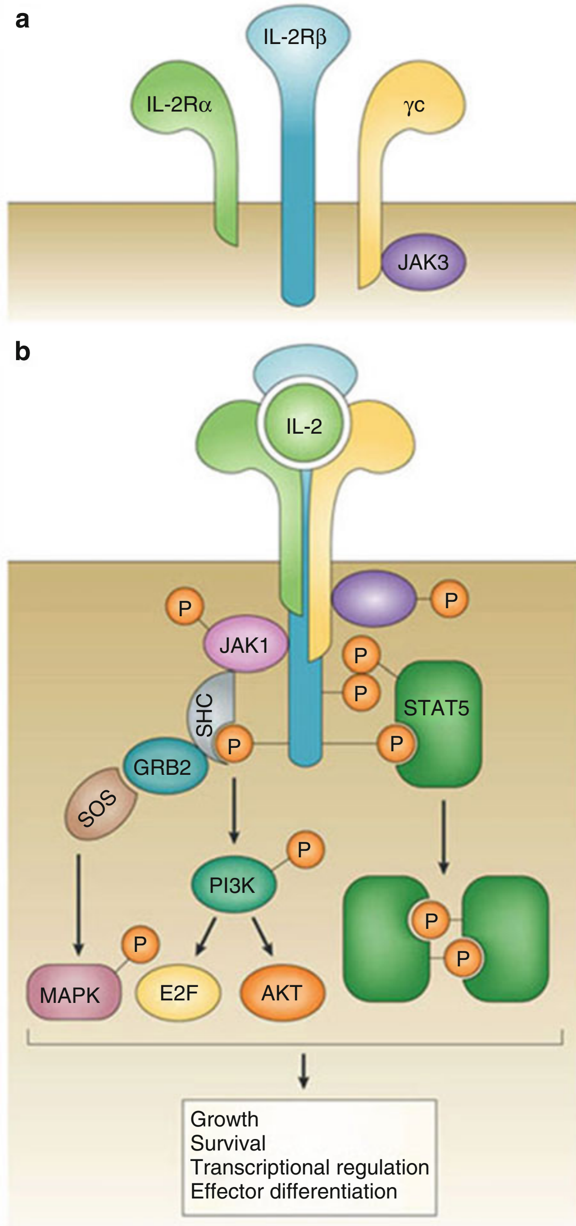

Fig. 2.1 Depiction of three chains of IL-2 receptors: the high-affinity IL-2 R is composed of three chains, α, β, and ϒ. The β and ϒ chains are responsible for signal transduction (Reproduced with permission. Source: Malek TR, Bayer AL (2004) Tolerance, not immunity, crucially depends on IL-2. *Nature Reviews Immunology*. 4: 665–6674. Nature Publishing Group)

Table 2.2 IL-2 receptors

	Low affinity	Intermediate affinity	High affinity
Affinity constant	10^{-8} M	10^{-7} M	10^{-11} M
Dissociation constant	10^{-8} M	10^{-9} M	10^{-11} M
Subunits	IL-2Rα	IL-2Rβγ	IL-2Rαβγ

trimeric IL-2Rαβγ is the high-affinity receptor (Table 2.2). The α chain is not expressed on resting T cells but only on activated T cells and is also called TAC (T-cell activation) receptor. Both β and γ chains are required for the signal transduction mediated via IL-2 receptors. The low-affinity and high-affinity IL-2 receptors are expressed by activated $CD4^+$ and $CD8^+$ T cells and in low numbers on activated B cells. The intermediate affinity IL-2 receptors are expressed on natural killer cells and in low numbers on resting T cells.

When IL-2 binds to high-affinity receptors, it becomes internalized following receptor-mediated endocytosis. After high-affinity binding, there is an increase in the stimulation of phosphoinositol turnover, redistribution of protein kinase C from the cytoplasm to cell membrane, and an increased expression of IL-2 receptors, with low-affinity receptors being preferentially increased.

2.4.2 Clinical Use for IL-2

2.4.2.1 Immunotherapy for Cancer

Proleukin (Aldesleukin)

Proleukin is recombinant human IL-2 that received approval for the treatment of renal cell carcinoma in 1992 and for the treatment of metastatic melanoma in 1998. The therapy is restricted to patients with normal cardiac and pulmonary functions. The treatment generally consists of two treatment cycles, each lasting for 5 days and separated by a rest period. Every 8 h, a dose of 600,000 IU/kg (0.037 mg/kg) is administered. The IV infusion period is 15 min and a maximum of 14 doses are administered. After a rest period of 9 days, another 14 doses are administered. Additional treatment can be given following an evaluation after 4 weeks.

The most frequent adverse reactions associated with administration of proleukin include fever, chills, fatigue, malaise, nausea, and vomiting. It has also been associated with capillary leak syndrome (CLS). CLS is defined as a loss of vascular tone and effusion of plasma proteins and fluids into the extravascular space. This leads to hypotension and decreased organ perfusion, which may cause sudden death. Other side effects include anaphylaxis, injection site necrosis, and possible autoimmune and inflammatory disorders.

LAK Therapy

IL-2 has been tested for antitumor effects in cancer patients as part of LAK therapy. LAK (lymphokine-activated killer cells) therapy involves infusion into cancer patients of their own (autologous) lymphocytes after they have been treated in vitro with IL-2 for a minimum of 48 h to generate LAK cells. IL-2 needs to be administered with LAK cells in doses ranging from 10^3 to 10^6 U/m^2 of body area or from 10^4 to 10^5 U/kg body weight.

IL-2 and AIDS

The human immunodeficiency virus (HIV) is a retrovirus that infects CD4$^+$ cells. After HIV becomes integrated into the genome of the CD4$^+$ cells, activation of these cells results in the replication of virus, which causes lysis of the host cells. Patients infected with HIV, and with AIDS, generally have reduced numbers of helper T cells, and the CD4:CD8 ratio may be as low as 0.5:1 instead of the normal 2:1. As a consequence, very little IL-2 is available to support the growth and proliferation of CD4$^+$ cells despite the presence of effector cell, B cells, and cytolytic T cells. Proleukin in combination with anti-retroviral therapy increases the number of CD4$^+$ cells. Low-frequency doses of subcutaneous proleukin at intervals maintained increase CD4$^+$ cell levels. The CD4 count increased to 1005 cells/μL from 520 cells/μ L and the mean of CD4$^+$ cells presents from 27 to 38%. However, the increase in CD4$^+$ T cells did not result to lowering the risk of opportunistic infections and death associated with HIV infection.

2.4.3 Denileukin Diftitox (Ontak)

Denileukin diftitox is a drug that is used to treat cancer. It is a combination of recombinant IL-2 and diphtheria toxin. The drug binds to IL-2 receptor via IL-2 and allows the entrance of diphtheria toxin into the cell. This results in the killing of those cells, which express IL-2 receptors. Certain malignant leukemias and lymphomas express these receptors and are the targets of denileukin diftitox. It is used to treat cutaneous T-cell lymphoma. In a trial of stage IV cutaneous T-cell lymphoma, denileukin diftitox extended the life expectancy from 8 months to more than a year. Its side effect may include vision loss.

2.5 IL-15

IL-15 is structurally similar to IL-2 and is characterized as T-cell growth factor. It belongs to the four α- helix bundle family of the cytokines. Despite the presence of IL-15 mRNA in many tissues, it is secreted as a mature protein predominantly by

macrophages, monocytes, and dendritic cells. Many agents, including cytokines, can induce the expression of IL-15. GM-CSF and interferon-γ induce its expression. Other gents include double-stranded mRNA, lipopolysaccharides through Toll-like receptors, unmethylated CpG oligonucleotides, and infection of monocytes. IL-15 is a pleiotropic cytokine, which is important for both innate and acquired responses, and is constitutively expressed on macrophages, monocytes, dendritic cells, fibroblasts, nerve cells, and keratinocytes. After binding to its receptors, IL-15 sends signals via an IL-2/IL-15β receptor chain (CD122) and the common γ chain (γ-C CD132). It is produced by mononuclear phagocytes after viral infection.

IL-15 produces its effect via juxtacrine signaling, including intracrine and reverse signaling. It exists in soluble as well as membrane-bound forms. The majority of this cytokine is present in membrane-bound form, where it is present on the cell membrane or presented by IL-15Rα. Trans-presentation is the mechanism through which this cytokine acts, and this process is transduced via membrane-bound complex IL-15/IL-15Rα. It can bind to IL-15Rβγ signaling complex with lower affinity or binds to IL-15Rα alone with high affinity. Its binding to IL-15β subunit activates Janus kinase 1 and binding to γ subunit induces Janus kinase 2. This results in the phosphorylation of induction of signal transducer and activator of transcription (STAT)-3 and STAT5. Since IL-2 and IL-15 share receptor subunits, their downstream effects are similar. Downstream pathways, which are activated, include MAPK (Mitogen-activated protein kinase), Bcl-2 (B-cell lymphoma), Lck (lymphocyte-activated protein kinase), and Syk (spleen tyrosine kinase). The ultimate effect of signal transduction is proliferation and maturation of cells. Its signaling pathway in mast cells is different and involves JAK2 and STAT5.

The regulation of induction and proliferation of T cells and natural killer cells is regulated by IL-15. Furthermore, IL-15 is responsible for the maintenance of memory T cells through survival signals and the development of natural killer cells. IL-15 and IL-2 negatively regulate each other, because they share some subunits of their receptors. The balance between IL-15 and IL-2 controls the proliferation and survival of CD8+ memory T cells. Since IL-15 augments tumor-killing effector function of CD8+ T cells, a clinical trial is underway to assess the safety and efficacy of this cytokine to treat metastatic melanoma and renal cell carcinoma.

2.6 Interleukin-4

Interleukin-4 is a pleiotropic cytokine produced by TH2 cells, mast cells, and natural killer cells. Other specialized subsets of T cells, basophils, and eosinophils also produce IL-4. It regulates the differentiation of antigen-activated naïve T cells. These cells then develop to produce IL-4 and a number of other TH2 type cytokines including IL-5, IL-10, and IL-13. IL-4 suppresses the production of TH2 cells. It is required for the production of IgE and is the principal cytokine that causes isotype switching of B cells from IgG expression to IgE and IgG4. As a consequence, it regulates allergic disease. IL-4 leads to a protective immunity against helminths and

other extracellular parasites. The expression of MHC class II molecules on B cells and the expression of IL-4 receptors is upregulated by IL-4. In combination with TNF, IL-4 increases the expression of vascular cell adhesion molecule-1 (VCAM-1) and decreases the expression of E-selectin, which results in eosinophil recruitment in lung inflammation.

IL-4 mediates its effects via specific IL-4 receptors, which are expressed on a number of tissues including hematopoietic cells, endothelium, hepatocytes, epithelial cells, fibroblasts, neurons, and muscles. The receptor is composed of an α chain that is the high-affinity receptor, but its signaling requires a second chain, a γ chain (γC), which is also a component of IL-2 receptors. However, the presence of a γ chain does not significantly increase the affinity of the receptor complex for IL-4. IL-4 causes the heterodimerization of the α chain with the γ chain, resulting in IL-4 receptor-dependent signaling pathway. As is the case with other cytokines, the signaling pathways activated after binding of IL-4 to its receptors are insulin receptor substrate (IRS-1/2) and Janus family tyrosine kinases – signal transducers and activators of transcription (JAK-STAT) pathways. However, for IL-4, the specificity results from the activation of STAT6.

2.7 Interleukin-5

Interleukin-5 is secreted predominantly by TH2 lymphocytes. However, it can also be found in mast cells and eosinophils. It regulates the growth, differentiation, activation, and survival of eosinophils. IL-5 contributes to eosinophil migration, tissue localization, and function and blocks their apoptosis. Eosinophils play a seminal role in the pathogenesis of allergic disease and asthma and in the defense against helminths and arthropods. The proliferation and differentiation of antigen-induced B lymphocytes and the production of IgA is also stimulated by IL-5. TH2 cytokines, IL-4 and IL-5, play a central role in the induction of airway eosinophilia and AHR. It is a main player in inducing and sustaining the eosinophilic airway inflammation.

IL-5 mediates its biological effects after binding to IL-5R, which is a membrane-bound receptor. The receptor is composed of two chains, a ligand-specific α receptor (IL-5R α) and a shared β receptor (IL-5Rβ). The β chain is also shared by IL-3 and granulocyte-monocyte-colony-stimulating factor (GM-CSF), resulting in overlapping biological activity for these cytokines. The signaling through IL-5R requires receptor-associated kinases. Two different signaling cascades associated with IL-5R include JAK-STAT and Ras/MAPK pathways.

IL-5 is usually not present in high levels in humans. However, in a number of disease states where the number of eosinophils is elevated, high levels of IL-5 and its mRNA can be found in the circulation, tissue, and the bone marrow. These conditions include the diseases of respiratory tract, hematopoietic system, gut, and skin. Some other examples include food and drug allergies, atopic dermatitis, aspirin sensitivity, and allergic or nonallergic respiratory diseases.

Another way of interfering with IL-5 or IL-5R synthesis is by the use of antisense oligonucleotides. Antisense oligonucleotides are short synthetic DNA sequences that can hybridize specifically to the mRNA of the cytokine or its receptors. This will result in the inhibition of the transcription and processing of mRNA. The administration of IL-5-specific antisense oligonucleotides results in reduced lung eosinophilia in animal models. However, there is not a complete inhibition of antigen-specific late-phase airway hyperresponsiveness, suggesting that in addition to IL-5, other pathways may also be involved in airway hyperreactivity.

2.8 Interleukin-6

Interleukin-6 is a pro-inflammatory cytokine, which is a member of the family of cytokines termed “the interleukin-6 type cytokines.” The cytokine affects a variety of processes including the immune response, reproduction, bone metabolism, and aging. IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to trauma, burns, tissue damage, inflammation, IL-1 and, to a lesser extent, TNF-α. Pathogen-associated molecular pattern (PAMP) binding to the Toll-like receptors (TLR) present on macrophage results in the release of IL-6. This cytokine is synthesized by some activated T cells as well. It is also secreted by osteoblasts to stimulate osteoclast formation. Acute phase response and fever is produced by IL-6, which is also the case for IL-1 and TNF-α. It affects differentiation of B cells and causes neutrophil mobilization. IL-6 is elevated in patients with retroviral infection, autoimmune diseases, and certain types of benign or malignant tumors. It stimulates energy mobilization in the muscle and fatty tissue resulting in an increase in body temperature. IL-6 acts as a myokine – cytokine produced by muscles – and muscle contraction results due to elevated IL-6 concentrations. The expression of IL-6 is regulated by a variety of factors, including steroidal hormones, which could be at both the transcriptional and posttranscriptional levels. IL-6 mediates its effects via binding to cell surface receptors, IL-6R, which are active in both membrane-bound and soluble forms.

2.9 Interleukin-9

Originally described as a mast cell growth factor due to its ability to promote the survival of primary mast cells and as an inducer of IL-6 production, IL-9, which is secreted by TH9 cells, stimulates the release of a number of mediators of the mast cells and promotes the expression of the high-affinity IgE receptors (FcER1α). IL-9 augments TH2-induced inflammation and enhances mucus hypersecretion, and the expression of its receptors is increased in asthmatic airways. It also promotes eosinophil maturation in synergy with IL-5. IL-9 activates airway epithelial cells by stimulating the production of several chemokines, proteases, mucin genes, and ion

channels. It is important to point out that as opposed to the IL-4-induced isotype switching and production of IgE, or the IL-5 mediated stimulation of eosinophil maturation, IL-9 induces actions of other cytokines. It is an essential cytokine for asthmatic disease as biopsies from asthmatic patients show an increase in the expression of IL-9 as compared to healthy individuals, and therefore it is an important therapeutic target for clinical intervention.

2.10 Interleukin 10

First identified as an inhibitor of IFN-γ synthesis in TH1 cells, IL-10 is an important immunoregulatory cytokine. It is an anti-inflammatory cytokine that was first called human cytokine synthesis inhibitory factor. IL-10 is secreted by macrophages, TH2 cells, and mast cells. Cytotoxic T cells also release IL-10 to inhibit viral infection-stimulated NK cell activity. IL-10 is a 36 KD dimer composed of two 160 long chain amino acid residue. Its gene is located in chromosome 1 in human and consists of 5 exons. IL-10 inhibits the synthesis of a number of cytokines involved in the inflammatory process including IL-2, IL-3, GM-CSF, TNF-α, and INF-γ. Based on its cytokine-suppressing profile, it also functions as an inhibitor of TH1 cells, and by virtue of inhibiting macrophages, it functions as an inhibitor of antigen presentation. Interestingly, IL-10 can promote the activity of mast cells, B cells, and certain T cells.

There are several viral IL-10 homologues: Epstein–Barr virus (BCRF.1), cytomegalovirus, herpes virus type 2, orf virus, and Yaba-like disease virus. Now interleukin-10 family of cytokines include not only IL-10 but also its viral gene homologues and several other cytokines including IL-19, IL-20m, IL-22, IL-24, IL-26, IFN-λ1, IFN-λ2, and IFN-λ3. IL-10 mediates its effects after binding to two receptor chains, IL-10R1 (α) and IL-10R2 (β). These receptors are the members of class II or interferon receptor family. The interaction of IL-10 with its receptors is highly complex and IL-10R2 (β) chain is essential for the production of its effects. Several hundred genes are activated after interaction of IL-10 with its receptors. The tyrosine kinases JAK1 and TyK2 are activated by the interaction of IL-10 with its receptors, which results in the induction of transcription factors1, STAT3, and STAT5 and eventual gene activation.

The major immunobiologic effect of IL-10 is the regulation of the TH1/TH2 balance. TH1 cells are involved in cytotoxic T-cell responses, whereas TH2 cells regulate B-cell activity and function. IL-10 is a promoter of TH2 response by inhibiting IFN-γ production from TH1 cells. This effect is mediated via the suppression of IL-12 synthesis in accessory cells. IL-10 is involved in assisting against intestinal parasitic infection and local mucosal infection by co-stimulating the proliferation and differentiation of B cells. Its indirect effects also include the neutralization of bacterial toxins.

IL-10 is a potent inhibitor of IL-1, IL-6, IL-10 itself, IL-12, IL-18, CSF, and TNF. It not only inhibits the production of pro-inflammatory mediators but also aug-

ments the production of anti-inflammatory factors including soluble TNF-α receptors and IL-1RA. IL-10 downregulates the expression of MHC class II molecules (both constitutive and IFN-γ-induced), as well as that of costimulatory molecules, CD86, and adhesion molecule, CD58. It is an inhibitor of IL-12 production from monocytes, which is required for the production of specific cellular defense response. IL-10 enhances the expression of CD16, CD32, and CD64 and augments the phagocytic activity of macrophages. The scavenger receptors, CD14 and CD163, are also upregulated on macrophages by IL-10. It is a stimulator of NK cells, enhances their cytotoxic activity, and also augments the ability of IL-18 to stimulate NK cells. Based on its immunoregulatory function, IL-10 and ligands for its receptors are tempting candidates for therapeutic intervention in a wide variety of disease states, including autoimmune disorders, acute and chronic inflammatory diseases, cancer, infectious disease, psoriasis, and allergic disease. However, to date, the results of its clinical trials as a supplement in Crohn's disease have been disappointing.

2.11 Interleukin-22

IL-22, a member of IL-10 superfamily, is a mediator of inflammatory responses. It is synthesized by a variety of cells including $CD4^+$ T cells (TH1, TH17, and TH22), $CD8^+$ T cells, γδ T cells, natural killer T (NKT) cells, lymphoid tissue inducer (LTi) cells, certain NK cell subsets, and activated dendritic cells. In epithelial cells, specifically GI and respiratory epithelial cells, it is responsible for innate immune response against bacteria and yeast. It plays roles in both the innate and acquired immune responses. IL-22 binds to IL-10R2 and IL-22R1 subunits. The IL-22 receptors are not expressed on immunocompetent cells, but on other tissue cells, including lung and intestinal epithelial cells, hepatocytes, pancreatic islets, and keratinocytes. Its expression is derived through STAT3 and retinoid orphan receptor ϒt (RORϒt). The cell signaling for IL-22 is achieved via the interferon receptor-related proteins, CRF2-4 and IL-22R. Binding of the agonist to the receptors activates JAK1 and Tyk2. The downstream signaling results from the activation of STAT3, Akt, and mitogen-activated protein kinases (MAPK). STAT1 and STAT5 may also be involved but to a lesser extent. After binding to its receptors and interacting with a soluble binding protein (IL-22 BP), IL-22 initiates and modulates immune response. It is involved in both the pro-inflammatory responses and tissue repair.

The antimicrobial effects of IL-22 can be divided into three phases: (1) It induces the proliferation of epithelial cells that form a barrier against invading pathogens. (2) In combination with other cytokines, it produces antimicrobial agents, including S100A7, S100A8, S100A9, β-defensin-2, β-defensin-3, RegIIIβ, RegIIIϒ, and protective mucus (Muc1, Muc3, Muc10, and Muc13). (3) IL-22 causes the production of IL-1β, IL-6, granulocyte colony-stimulating factor (G-CSF), lipopolysaccharide (LPS)-binding protein (LBP), and serum amyloid A (SAA), which are all

pro-inflammatory in nature. A prolonged or excessive production of IL-22 can cause psoriasis-like skin inflammation. Its pathogenic properties are augmented when it is released along with IL-17.

2.12 Interleukin-11

Interleukin-11, a member of the IL-6 super family, is produced by bone marrow stroma and activates B cells, plasmacytomas, hepatocytes, and megakaryocytes. The gene for IL-11 is located on chromosome 19. IL-11 induces acute phase proteins, plays a role in bone cell proliferation and differentiation, increases platelet levels after chemotherapy, and modulates antigen-antibody response. It promotes differentiation of progenitor B cells and megakaryocyte. The recovery of neutrophils is accelerated by IL-11 after myelosuppressive therapy. IL-11 also possesses potent anti-inflammatory effects due to its ability to inhibit nuclear translocation of nuclear factor-κB. Additional biological effects of this cytokine include epithelial cell growth, osteoclastogenesis, and inhibition of adipogenesis. The effects of IL-11 are mainly mediated via the IL-11 α receptor chain. IL-11 forms a high-affinity complex in association with its receptor and associated proteins and induces gp-130-dependent signaling.

2.12.1 Oprelvekin (Neumega)

Recombinant human interleukin-11 (oprelvekin) is a polypeptide of 177 amino acids. It differs from natural IL-11 due to lack of glycosylation and the amino terminal proline residue. Oprelvekin is administered by subcutaneous injection, usually 6–24 h after chemotherapy, at a dose of 25–50 μg/kg per day. The drug has a half-life of about 7 h. It is used to stimulate bone marrow to induce platelet production in non-myeloid malignancies in patients undergoing chemotherapy. The common side effects of oprelvekin include fluid retention, tachycardia, edema, nausea, vomiting, diarrhea, shortness of breath, and mouth sores. Other side effects include rash at the injection site, blurred vision, paresthesias, headache, fever, cough, and bone pain. Rarely, capillary leak syndrome may occur.

2.13 Interleukin-12

In response to antigenic stimulation, IL-12 is produced by dendritic cells, macrophages, and B-lymphoblastoid cells. It is made up of IL-12A (p35) and IL-12B (p40) subunits and produces its effects after binding to a heterodimer receptors, IL-12R, formed by IL-12R-β1 and IL-12R-β2. IL-12 plays a role in the

development of TH1 cells from naïve helper T cells. It causes the growth and effector function of T cells and is also referred as T-cell-stimulating factor. IL-12 induces the synthesis and release of IFN-ϒ and TNF-α, while inhibiting the effects of IL-4 on IFN-ϒ. It is crucial for the effector function of T cells and natural killer cells, as IL-12 augments the effector function of cytolytic T cells and natural killer cells. Its anti-angiogenic property is mediated through enhanced production of IFN-ϒ.

2.14 Interleukin-35

IL-35 belongs to IL-12 family cytokines, which is produced by regulatory but not effector T cells. It is a dimeric protein that has two chains, IL-12α and IL-27β, and is immunosuppressive in nature. IL-35 is not constitutively expressed in tissues, but pro-inflammatory agents cause the transcription of genes encoding it in monocytes, vascular endothelial cells, and smooth muscle cells. It augments the proliferation of Treg cells and attenuates the function of TH17 cells.

2.15 Interleukin-13

IL-13 belongs to the same α-helix super family as IL-4, and their genes are located 12Kb apart on chromosome 5q31. It was originally identified for its effects on B cells and monocytes, which included isotype switching from IgG to IgE, inhibition of inflammatory cytokines, and enhancement of MHC class II expression. Initially, IL-13 appeared similar to IL-4 until its unique effector functions were recognized. Nevertheless, IL-13 and IL-4 have a number of overlapping effects. IL-13 also plays an essential role in resistance to most gastrointestinal nematodes.

It regulates mucus production, inflammation, fibrosis, and tissue remodeling. IL-13 is a therapeutic target for a number of disease states including asthma, idiopathic pulmonary fibrosis, ulcerative colitis, cancer, and others. Its signaling is mediated via IL-4 type 2 receptor. The receptor consists of IL-4Rα, IL-13Rα1, and IL-13Rα2 chains.

IL-13 induces physiologic changes in organs infected with parasites that are essential to get rid of the invading pathogen. In the gut, it induces a number of changes, which makes the surrounding environment of the parasite less hospitable, such as increase contractions and hypersecretion of glycoproteins from gut epithelial cells. This results in the detachment of the parasites from the wall of the gut and their subsequent removal. IL-13 response in some instances may not resolve infection and may even be deleterious. For example, IL-13 may induce the formation of granulomas after organs such as the gut wall, lungs, liver, and central nervous system are infected with the eggs of *Schistosoma mansoni*, which may lead to organ damage and could even be life-threatening.

IL-13 is believed to inhibit TH1 responses, which will inhibit the ability of the host to get rid of the invading pathogens. The role of IL-13 in the etiology/pathogenesis of allergic disease/asthmas has drawn broad attention. It induces airway hyperresponsiveness and goblet cell metaplasia, which result in airway obstruction and cause allergic lung disease. IL-13/chemokine interactions play a key role in the development of airway hyperresponsiveness and mucus production. IL-13 induces the expression of chemokine, eotaxins. These chemokines recruit eosinophils into the site of inflammation in synergy with the IL-5. Eosinophils release IL-13 and induce the production of IL-13 from TH2 cells, which is mediated via IL-18. IL-13 subsequently through its effects on epithelial and smooth muscle cells aids in the development of AHR and mucus production. In addition to its potent activation of chemokines, IL-13 also is an inducer of adhesion molecules involved in asthma.

2.16 Interleukin-17

IL-17, secreted by TH17 cells and induced by IL-23, is involved in delayed-type reactions. It is a pro-inflammatory cytokine that is secreted in response to the recognition of invading pathogen by the immune system. One of its functions is the destruction of the cellular matrix of the invading pathogen. IL-17 increases chemokine production resulting in the recruitment of neutrophils and monocytes to the site of injury/inflammation. It causes tissue damage in delayed-type reactions. The mechanism of action of IL-17 is in synergism with IL-1 and TNF-α. The cytokine is also associated with allergic disease and asthma. It mediated its effects via a family of receptors IL-17RA, IL-17RB, and IL-17RC. IL-17 inhibiting monoclonal antibody is used to treat psoriasis. The inhibitors of IL-17 are under investigation to treat a number of disorders including autoimmune diseases and inflammatory bowel disease.

2.17 Interleukin-18

IL-18 is a member of IL-1 family that promotes production of a variety of pro-inflammatory mediators and plays a role in cancer and various infectious diseases. It was originally identified as IFN-γ-inducing factor and is produced by cells of both hematopoietic and non-hematopoietic lineages, including macrophages, dendritic cells, intestinal epithelial cells, synovial fibroblasts, keratinocytes, Kupffer cells, microglial cells, and osteoblasts. The production of IL-18 is structurally homologous to IL-1β; it is produced as an inactive precursor of 24-kD, which lacks a signal peptide. Endoprotease IL-1β-converting enzyme activates it after cleaving pro-IL-18, resulting in a biologically active cytokine. Caspase-1 plays an important role in the processing of IL-18 but is not exclusive since proteinase 3 can also perform the same function.

IL-18 augments T- and NK cell maturation, cytotoxicity, and cytokine production. It stimulates TH differentiation; promotes secretion of TNF-α, INF-γ, and GM-CSF; and enhances NK cell cytotoxicity by increasing FasL expression. IL-8-mediated neutrophil chemotaxis is promoted by IL-18 via its effects on TNF-α and INF-γ, which are stimulatory. It plays an important role in maintaining synovial inflammation and inducing joint destruction in rheumatoid arthritis. In synovium of patients with rheumatoid arthritis, enhanced levels of TNF-α and IL-1 are associated with augmented expression of IL-18.

IL-18 also induces IL-4, IL-10, and IL-13 production, increases IgE expression on B cells, and, in association with IL-2, enhances stimulus-induced IL-4 production from TH2 cells. Bone marrow-derived basophils produce IL-4 and IL-13 in response to a stimulus from IL-18 to IL-3. IL-18 in combination with IL-12 induces IFN-γ from dendritic cells and bone marrow-derived macrophages. Adhesion molecules, ICAM-1 and VCAM-1, are induced by this cytokine on synovial fibroblasts and endothelial cells. It inhibits osteoclast formation via its induction of GM-CSF from T cells. The receptors of IL-18, IL18Rα, and IL18Rβ, share their signaling mechanisms via IL-1R family. TLR also share the downstream signaling pathway of IL-18 and are known to regulate IL-18 expression.

It plays a critical role in host defense against bacterial, viral, fungal, and protozoan infections. One predominant mechanism of action is its induction of hosts INF-γ production, which activates several effector pathways including nitric oxide production, resulting in the clearance of the invading pathogens. A role of IL-18 in robust TH1 responses against mycobacterium tuberculosis and mycobacterium avium has been suggested. For viral infections, the effects of IL-18 are not only mediated via INF-γ but also by activation of $CD8^{+}$ T cells. IL-12 and IL-15 also play a role in its effects in host defense, and as a mediator of inflammation, where IL-18 works in concert with other cytokines and their signaling pathways. Multiple checkpoints are involved in the modulation of inflammation by IL-18. IL-18-binding protein (IL-18BP) is the naturally occurring antagonist that serves as negative feedback mechanism for IL-18, as several isoforms of this antagonist have been identified.

2.18 Interleukin-23

IL-23 is a heterodimeric cytokine. It is composed of p19 unit (IL-23 subunit α) and p40 (IL-12 subunit β) of which p40 subunit is shared with IL-12. IL-23 is produced by dendritic cells and macrophages. It plays an important role in inflammatory responses against infection. The functional receptors for IL-23 are comprised of IL-12R β1 and IL-23R. It plays a role in both the innate and acquired immune response. IL-23 inhibits the infiltration of cytolytic T cells (CD8+) into tumors, enhances synthesis of the matrix metalloprotease MMP9, and induces angiogenesis. In combination with IL-6 and TGFβ, IL-23 induces naïve CD4+ T cells to produce TH17 cells, which as previously described are distinct from TH1 and TH2 cells.

TH17 cells secrete IL-17 that is a pro-inflammatory cytokine. IL-17 enhances T-cell priming and the production of IL-1, IL-6, TNF-α, NOS-2, as well as other cytokines. As a consequence, a pro-inflammatory response is produced.

2.19 Interferons

Although originally identified as proteins with antiviral activity, these inducible cytokines play an important role in regulating innate and acquired immunity. Initially, characterized by the secreting cell type, interferons are now divided into two groups, type I and type II interferons. Type I IFN, which are also called IFN-α/β family, are the product of numerous genes and include INF-α, INF-β, INF-ω, INF-κ, INF-ε, and INF-λ. Almost all cell types secrete type I IFN; however, hematopoietic cells are the major source of IFN-α and INF-ω, and fibroblasts are the major producers of IFN-β. Macrophage under appropriate induction also secretes IFN-β. Their structural genes are located on chromosome 9 in human. The type II interferon IFN-γ is the product of a single gene on chromosome 12 in human. The stimuli for the production of type I interferons are viral and microbial infections and double-stranded RNA.

2.19.1 Type I Interferons

Type I Interferons are two distinct groups of proteins, IFN-α (approx. 18 KD) and IFN-β (20 KD). IFN-α is subdivided into two subgroups, IFNα1 and IFNα2/IFN-ω. Viral infection is the most potent natural signal for the synthesis of type I interferons.

The principal biologic actions of type I interferons include inhibition of the viral replication, inhibition of cell proliferation, increase in the lytic potential of natural killer cells, and modulation of MHC molecule expression. They increase the expression of MHC class I molecules and decrease the expression of MHC class II molecules.

Type I interferons exert their biological effects after binding to distinct heterodimeric cell surface receptors on the target cells. Binding of the agonist to the cell surface receptors results in the activation of Janus-activated kinase (JAK)-STAT signal transducers and activators of transcription signaling pathway. The JAK-STAT activation results in the induction of specific genes. These genes contain IFN-specific response elements or IFN-γ-stimulated sequence. The interferons have both overlapping as well as distinct pharmacologic activities because some genes overlap partially, whereas some interferons are produced at different sites.

Interferon-α/interferon-β mediate antiviral activity by multiple mechanisms. A series of antiviral proteins are produced after interferon-α/interferon-β binds to its specific cell surface receptors. The proteins induced by interferons include a

2′–5′-oligoadenylate synthetase and a protein kinase; both in the presence of double-stranded RNA can inhibit protein synthesis. A latent cellular endoribonuclease is activated by adenylate oligomers produced by an oligoadenylate synthetase, which breaks down viral as well as cellular single-stranded RNAs. The protein kinase inactivates EIF-2 (eukaryotic initiation factor) after phosphorylation, which is involved in protein synthesis and is also an effector for apoptosis. Furthermore, peptide elongation is prevented as a result of cleaving of transfer RNA by a phosphoesterase that is induced by interferon-α/β. Depending on the family of the virus, multiple steps may be inhibited by interferon with varying degrees.

2.19.1.1 Clinical Applications of Interferons

Interferon-α

Interferon-α may be used for the treatment of condylomata acuminata (venereal or genital warts); malignant melanoma; hairy cell leukemia; hepatitis B and C; and other types of cancer including skin, kidney, and bone cancer.

Interferon Alpha-2a (Roferon A)

Produced by recombinant DNA technology, interferon alpha-2a is used for the treatment of chronic myeloid leukemia, Kaposi's sarcoma, lymphoma, hairy cell leukemia, hepatitis B or C, and cancer of the skin and kidney. It can only be administered by injection or into the blood stream and the most common form is the subcutaneous injection. This cytokine can be injected every day; however, commonly it is administered three times a week. The antiviral or antitumor activity of interferon-α-2a is mediated via its inhibition of viral replication and modulation of host immune response as well as its antiproliferative activity. It is filtered through the glomeruli, and its proteolytic degradation takes place during tubular reabsorption. The common side effects include flu-like symptoms of fever, fatigue, chills, dry mouth, GI disorders, changes in mood, and temporary effects on the bone marrow. The occasional side effects may include skin rash, hair thinning, loss of appetite, and loss of fertility.

Peginterferon α -2a

Pegylated alpha interferon is made by attaching polyethylene glycol (PEG) to the α-interferon. PEG is a large water-soluble molecule that decreases the clearance of α-interferon and also increases the duration of its activity. This modified cytokine is used to treat chronic hepatitis C. However, it is rarely used as a single therapeutic agent for hepatitis C because of its low response rate.

Interferon-α-2b

Interferon-α-2b is a water-soluble alpha interferon protein produced by recombinant DNA technology. Both interferon-α-2b and α-2a are pure clones of single interferon subspecies, but they differ by the virtue of two amino acids. The potencies of both α-2a and α-2b interferons are similar. Interferon-α-2b is also available in pegylated form. All interferon-α cytokines augment the killing of target cells by lymphocytes and inhibit the replication of virus in infected cells.

Interferon-β

Natural interferon-β is predominantly synthesized by fibroblasts. Its sequence is 30% homologous to that of interferon-α. The receptors for both interferon-α and β are the same but the fit of the receptor is different for the two agonists. There are also differences in structure (INF-β is glycosylated on one site), pharmacokinetics and binding to tissues between interferon-α and β.

Interferon-β -1a is used to treat patients with a relapsing form of multiple sclerosis (MS). It is not a cure for MS; however, it may slow some disabling effects of the disease. Interferon-β -1a may also decrease the number of relapses of MS. The possible mechanisms of action for the treatment of MS may include the antagonism of IL-4 and interferon-γ. It also modifies the mechanics of blood barrier since it inhibits cell adhesion, cell migration, and metalloproteinase activity. INF-β induces IL-10 and TGFβ, which are anti-inflammatory cytokines. It is also used for the treatment of genital warts.

2.19.2 Type II Interferons

2.19.2.1 Interferon-γ

IFN-γ modulates a number of the components of the immune response. This is the only type II interferon as compared to more than twenty types of type I interferons (INF-α, INF-β, INF-ω, and INF-tau). It is not related to type I interferons, has separate receptors, and is encoded by a different chromosomal locus. INF-γ is produced by activated T lymphocytes (TH1 and $CD8^+$ cells), natural killer cells, B cells, NKT cells, and professional antigen-presenting cells. It promotes the activity of cytolytic T lymphocytes, macrophages, and natural killer cells. The cell self-activation and activation of nearby cells in part may result from INF-γ production by professional antigen-presenting cells, which include monocyte/macrophage and dendritic cells. The early host defense against infection is likely to utilize INF-γ secreted by NK and professional antigen-presenting cells. In acquired immune responses, T lymphocytes are the major source of INF-γ.

INF-γ production is regulated by IL-12 and IL-18, both cytokines secreted by antigen-presenting cells. In the innate immune response, a link is established between infection and INF-γ by these cytokines. IL-12 and chemokines including MIP-1α (macrophage inflammatory protein-1α) are secreted as macrophage recognizes pathogens, and NK cells are attracted to the site of inflammation by the chemokines. This is followed by the induction of INF-γ production and secretion by IL-12. IL-12 and IL-18 further stimulate the production of INF-γ from macrophages. The production of INF-γ is inhibited by IL-4, IL-10, and TGF-β.

IFN-γ is a potent activator of mononuclear phagocytes. The expression of both MHC class I and class II molecules is augmented by INF-γ as IFN-γ-induced upregulation of MHC class I molecules is pivotal for host defense against intracellular pathogens, resulting in an increased susceptibility to cytolytic T cells for recognition and consequent promotion of cell-mediated immune response. The stimulation by IFN-γ results in the addition of "immuno proteosome subunits" and the removal of constitutive proteosome subunits. The unstimulated cells contain β_1, β_2, and β_5 proteosome enzymatic subunits, which are encoded outside the MHC locus. β_1 is replaced by LMP2, β_2 by MECL, and β_5 by LMP7, and the expression of new subunit is stimulated by INF-γ. This results in the formation of new subunits of proteosomes. This is a potential mechanism utilized by IFN-γ to enhance the characteristics of peptides for MHC class I loading. The ability to immunoproteosome to cleave peptides enhances the ability of antigen fragments to bind to MHC class I molecules. The diversity of the antigenic fragments is increased, resulting in better immune surveillance. IFN-γ also augments the MHC class II antigen-presenting pathway and results in the activation of $CD4^+$ T cells via peptides. It not only stimulates the expression of MHC class II molecules on cells that constitutively express these antigens but also induce their expression on cells that do not constitutively express their genes. The expression of several other molecules including Ii chain; cathepsins B, H, L, and lysosomal proteases; and HLA-DM is upregulated by INF-γ. These molecules are involved in various processes associated with antigen presentation, peptide accessibility, and peptide loading.

IFN-γ is produced by TH1 cells and shifts the response toward a TH1 phenotype. This is accomplished by activation of NK cells which promotes innate immunity, augmenting specific cytolytic response and induction of macrophages. The induction of cytotoxic immunity can be direct or indirect via suppression of TH2 response. Another direct effect of IFN-γ is the differentiation of naïve $CD4^+$ lymphocytes toward a TH1 phenotype. The cytokines present are very important in this differentiation process. Furthermore, induction of IL-12 and suppression of IL-4 by IFN results in differentiation toward TH1 phenotype.

IFN-γ is an inhibitor of cell growth and proliferation. The proliferation is inhibited by augmenting the levels of Cip/Kip, CKIs, and Ink4. It increases p21 and p27 CKIs which inhibit the function of cyclin E, CDK2, and cyclin D, CDK4, respectively. This results in stopping the cell cycle at G1/S interphase. INF-γ induces apoptosis via activation of STAT1, which results in the production of large amounts of IRF-1 (interferon regulatory factors). Apoptosis may be needed to kill the invading pathogen-infected macrophages.

IFN-γ also induces the costimulatory molecules on the macrophages, which increases cell-mediated immunity. As a consequence, there is activation and increase in the tumoricidal and antimicrobial activity of mononuclear phagocytes, granulocytes, and natural killer cells. The activation of neutrophils by interferon-γ includes an increase in their respiratory burst. INF-γ stimulates the cytolytic activity of natural killer cells. It is an activator of vascular endothelial cells, promoting $CD4^+$ T-lymphocyte adhesion and morphological alterations, which facilitates lymphocyte extravasation. IFN-γ promotes opsonization by stimulating the production of IgG subclasses, which activate the complement pathway. A summary of the characteristics of some cytokines is shown in Table 2.3.

2.19.3 Type III Interferons

2.19.3.1 Interleukin-28

IL-28 is a cytokine that has two isoforms, IL-28A and IL-28B, and belongs to the type III interferon family. As opposed to most type-1 interferons, which are encoded by a single exon, IL-28 is encoded by multiple exons. It is involved in immune response against viruses. IL-28 induces an "antiviral state" via activation of Mx proteins, 2′5′-oligoadenylate synthetase and interferon-stimulated gene factor 3 (ISGF3G). It plays a role in the acquired immune response. In animal models, when administered as an adjuvant to vaccines, IL-28 enhances antigen-specific interferon-ϒ release and effector function of $CD8^+$ T cells. Its receptors are comprised of a unique IL-28 α receptor chain in combination with IL-10 β receptor chain. The response to interferon and ribavirin for the treatment of hepatitis C can be predicted by a single nucleotide polymorphism (SNP) near the IL-28 B gene, which is clinically relevant.

2.19.3.2 Interleukin-29

IL-29 also known as IFNλ1 is a type III interferon and is very similar to IL-28 in its amino acid sequence. It is involved in immune response against invading pathogens. IL-29 gene is upregulated in virally infected cells.

2.20 Colony-Stimulating Factors

A major cause of morbidity and mortality in patients who receive cytotoxic treatment or radiotherapy for cancer is bacterial and fungal infections. Intensive chemotherapy is associated with fever and infection, and the development of neutropenia further increases this risk of infection. Consequently, maximum doses of some

Table 2.3 Characteristics of selected cytokines

Name	Source	Target	Biological role
IL-1 (IL-1α and β)	Macrophages, dendritic cells, endothelial cells, other cells	TH and B cells and various other tissues	Activation (other details provided in the text)
IL-2	TH_1 cells	TH, T_C, and NK cells	T cell and NK proliferation, and induction of activity
IL-3	TH_1 and TH_2 cells, mast cells, NK cells	Hematopoietic and mast cells	Progenitor cell proliferation and differentiation
IL-4	TH_2 cells, mast cells, NK cells	B cells, T cells, mast cells, macrophages	Proliferation, isotype switching, induction of MHC class II expression
IL-5	TH_2 cells, mast cells	Eosinophils	Proliferation and differentiation
IL-6	Macrophages, TH_2 cells	Plasma cells, B cells and others	Differentiation and antibody secretion
IL-8	Bone marrow, thymus (stromal cells)	Neutrophils	Chemoattractant
IL-9	TH_2 cells	TH cells, mast cells, eosinophils	Induces inflammatory responses
IL-10	TH_2 cells	Macrophages, APC	Anti-inflammatory cytokine inhibits cytokine production
IL-11	Bone marrow (stromal cells)	B cell progenitors and others	Differentiation
IL-12	Macrophages, B cells	T_C, NK, and LAK cells	Proliferation and differentiation in synergy with IL-2
IL-13	TH cells	Macrophage B cells	Inhibition of inflammatory cytokines regulates inflammation. Parasitic infections
IL-16	T_C cells	TH cells	Chemotaxis
IL-17	TH17 cells	Cytokines	Delayed-type reactions
		Chemokines	
IL-18	Hematopoietic and non-hematopoietic lineage cells	T Cells NK cells	Pro-inflammatory cytokine; IFN-γ-inducing factor
Interferon-α	Leukocytes		Inhibitor of viral replication
Interferon-β	Fibroblasts		Inhibitor of viral replication

(continued)

Table 2.3 (continued)

Name	Source	Target	Biological role
Interferon-γ	TH_1, T_C, NK	A variety of cells including macrophages	Inhibitor of viral replication. Inhibitor of cell proliferation. Inhibitor of IL-4-induced isotype switching
TNF-α	Macrophages	Tumor cells, polymorphonuclear leukocytes, macrophages	Cytotoxicity, induction of cytokine secretion
TNF-β	T cells	Tumor cells, neutrophils, macrophages	Cytotoxicity, phagocytosis

cytotoxic drugs use are limited due to bone marrow toxicity. Higher doses of chemotherapy and radiation therapy have become possible due to a reduction in bone marrow damage with the availability of the colony-stimulating factors for clinical use.

The colony-stimulating factors are glycoproteins that support hematopoietic colony formation. They influence the survival, proliferation, and maturation of hematopoietic progenitor cells and regulate the activities of the mature effector cells. There are three lineage-specific CSF, granulocyte colony-stimulating factor (G-CSF), monocyte-macrophage colony-stimulating factor (M-CSF), erythropoietin, and two multipotential CSF, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

2.20.1 Clinical Uses of Colony-Stimulating Factors

The colony-stimulating factors prevent chemotherapy-induced neutropenia. They stimulate hemopoiesis in marrow failure. The colony-stimulating factors promote cell differentiation, assist in marrow transplantation, stimulate monocytes anticancer effects, and augment effector cell function.

2.20.2 Granulocyte Colony-Stimulating Factor (G-CSF)

Granulocyte colony-stimulating factor is a glycoprotein produced by macrophages, endothelium, and a variety of leukocytes. It stimulates the bone marrow to produce granulocytes and stem cells and then direct their migration from the bone marrow to the peripheral blood. G-CSF is a growth factor for the proliferation, differentiation, effector function, and survival of neutrophils. The gene for G-CSF is located on chromosome 17, locus q11.2–q12.

Granulocytes colony-stimulating factor mobilizes bone marrow-derived cells into the blood stream. These stem cells can migrate to ischemic myocardium and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. They may also induce metalloproteinases and vascular endothelial growth factor and thus play a role in tissue healing. Furthermore, GCF induces proliferation and enhanced survival of cardiomyocytes. This is accomplished via activation of G-CSF receptors in myocardium. G-CSF in association with TGF-β and collagen enhances ventricular expansion in the infarcted area.

Granulocyte colony-stimulating factor activates neutrophils, transforming them into the cells capable of respiratory burst and release of secretory granules. It also modulates the expression of adhesion molecules on neutrophils as well as CD11b/CD18 and plasma elastase antigen levels. G-CSF induces proliferation of endothelial cells, phagocytic activity of neutrophils, reactive oxygen intermediate production by neutrophils, and antibody-dependent cellular toxicity by neutrophils.

2.20.2.1 Filgrastim (Neupogen)

Recombinant human G-CSF (filgrastim) is a 175 amino acid glycoprotein. It differs from natural G-CSF due to a lack of glycosylation and having an extra N-terminal methionine. Pegylated recombinant human G-CSF (pegfilgrastim) is also available. Filgrastim administered to patients receiving cytotoxic chemotherapy for advanced cancer has resulted in a dose-dependent amelioration of neutropenia associated with cancer chemotherapy. It is well tolerated and may reduce the morbidity and mortality rate associated with chemotherapy, possibly permitting higher doses and a greater antitumor response. Filgrastim is also used after autologous stem cell transplantation to treat neutropenia. It reduces the duration of neutropenia and lessens morbidity secondary to bacterial and fungal infections. Additional use for this drug includes the treatment of severe congenital neutropenias, neutropenia in patients with AIDS resulting from treatment with Zidovudine, and patients donating peripheral blood stem cells for stem cell transplantation.

Filgrastim is administered by intravenous infusion or subcutaneous injection. The doses given are 1–20 μg/kg per day over at least a 30 min period. Generally a dose of 5 μg/kg is used in patients receiving chemotherapy for 14–21 days or longer. The half-life of the drug is 3.5 h. The side effects include bone pain, local skin reactions, and rarely cutaneous vasculitis.

2.20.3 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

Granulocyte-macrophage colony-stimulating factor is a glycoprotein produced by macrophages, T cells, mast cells, fibroblasts, and endothelial cells. It stimulates stem cells to produce neutrophils, monocytes, eosinophils, and basophils. Monocytes

migrating into tissue from the circulating blood differentiate into macrophages and undergo maturation.

2.20.3.1 Sargramostim (Leukine)

Recombinant human GM-CSF (Sargramostim) is a 127 amino acid glycoprotein, which is similar to natural GM-CSF except for variation in glycosylation and presence of a leucine in position 23. It has beneficial effects on bone marrow function in patients receiving high dose chemotherapy in the setting of autologous bone marrow transplantation as well as for the treatment of advanced cancers. Sargramostim is used in AIDS, myelodysplastic syndrome, and aplastic anemia where it stimulates bone marrow function. It has not shown beneficial effects in graft-versus-host disease but may be of value in patients with early graft failure. It has been used in patients donating peripheral blood stem cells because it mobilizes $CD34^+$ progenitor cells. Sargramostim is administered either by slow intravenous infusion or subcutaneous injection. The doses given are 125–500 $\mu g/m^2$ per day. Intravenous administration requires a period of at least 3–6 h. The half-life with subcutaneous injection is 2–3 h. The side effects with high doses include bone pain, flu-like symptoms, fever, diarrhea, nausea, and vomiting. Prolonged administration has produced marked weight gain, generalized edema, capillary leak, and hypotension. It also causes a dose-dependent, asymptomatic eosinophilia.

2.21 Tumor Necrosis Factor-Alpha (TNF-α)

This pro-inflammatory cytokine was first isolated in 1975, and its name is misleading in the sense that it does not cause the necrosis of all tumors. As a matter of fact, it may stimulate the growth of some tumors. TNF-α is a 185 amino acid glycoprotein, which is cleaved from a 212 amino acid peptide, and the cleavage occurs on the cell surface of mononuclear phagocytes. In humans, the genes for TNF-α are present on chromosome 7p21. The major cell source of TNF-α is the macrophage, specifically the endotoxin-activated mononuclear phagocyte. Other sources include endothelium after tissue damage, antigen-stimulated T cells, activated NK cells, and activated mast cells. IFN-γ augments TNF-α synthesis.

TNF-α is a mediator of both natural and acquired immunity. Local increasing concentrations of TNF-α cause heat, swelling, redness, and pain. TNF-α causes vascular endothelial cells to express new adhesion molecules. It increases the mobilization and effector function of neutrophils and their adhesiveness for endothelial cells. TNF-α induces the production of IL-1, IL-6, TNF-α itself, and chemokines via stimulation of macrophages. It exerts an interferon-like protective effect against viruses and augments expression of MHC class I molecules. TNF-α is an endogenous pyrogen that acts on cells in hypothalamic regulatory regions of the brain to induce fever. It suppresses appetite. The hypothalamic–pituitary–adrenal axis is

stimulated via the release of corticotropin-releasing hormone by TNF-α. It induces acute phase responses by activating hepatocytes. Acute phase proteins including C-reactive protein and mannose-binding protein (MBP) are detected in blood in response to an infection. It suppresses bone marrow stem cell division and reduces tissue perfusion by depressing myocardial contractility.

2.22 Tumor Necrosis Factor Receptors

There are two distinct types of TNF receptors, TNF-R1 (CD120a or P55) and TNF-R2 (CD120b or P75). They are implicated in inflammatory processes and both belong to the TNF receptor super family. TNF receptors are transmembrane proteins with intracellular domains which lack intrinsic enzymatic activity, and consequently, they require cytoplasmic proteins, which help initiate the receptor-induced signaling pathways. TNF-R1 possesses an intracellular death domain and TNF-R2 interacts with molecules of TNF receptor-associated factor 2 family (TRAF).

The receptors for TNF-α are widely distributed, although TNF-R1 is more common in non-hematopoietic cells. Both groups of receptors interact with the ligand TNF-α (soluble form) with similar affinity. As shown in Fig. 2.2, TNF-R1 recognizes both the membrane-bound and soluble TNF-α, whereas TNF-R2 binds to membrane-bound TNF-α with greater affinity. The signals initiated by two receptors are different since there are structural differences between the intracellular domains of two receptors. The activated TNF-R1 contains a death domain in its cytoplasmic region that recruits the adopter proteins. The downstream signaling involves different pathways which lead to cell death or survival.

After the binding of TNF-α to its receptors, there is induction of two major intracellular signaling pathways. One pathway leads to the transcription of other genes, and the other pathway leads to cell death or apoptosis. The two main transcription factors activated by TNF-α are AP-1 and NF-κB.

2.22.1 Etanercept (Enbrel)

Etanercept is a genetically engineered protein that is soluble TNF-α receptor. Its molecular weight is 75 kDa. It binds to TNF-α. It is used for the treatment of rheumatoid arthritis, juvenile rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis. Structurally, two TNF-α receptors are linked to an Fc portion of an IgG1 molecule. Consequently, an artificial antibody is constituted with two Fab sites, which are soluble human 75 kDa TNF-α receptors. It competitively inhibits the binding of TNF molecules to the TNF receptor sites. The binding of etanercept to TNF renders the bound TNF biologically inactive, resulting in the reduction of the inflammatory activity. The most frequent adverse side effects are injection site reactions, infections, and headache, and malignancies are rare. Etanercept is not

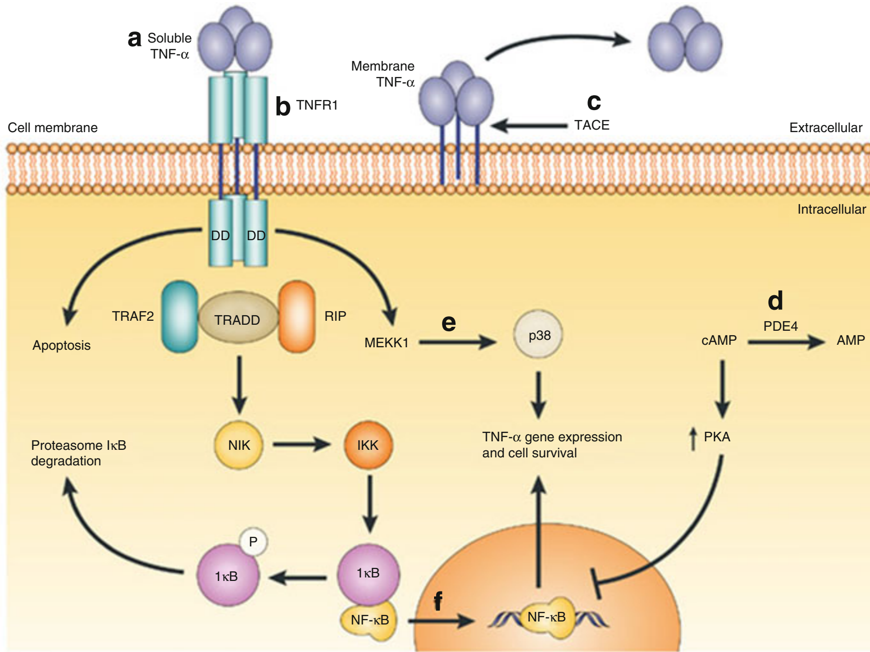

Fig. 2.2 TNF-α receptors and their signal transduction mechanisms: both membrane-bound and processed soluble TNF-α bind to TNF-α R1. Cell death or apoptosis is also a function of TNF-α R1. This is due to the presence of a death domain in the cytoplasmic region of TNF-α R1, which recruits the adopter proteins. Different pathways are involved in downstream signaling that leads to the death or survival of the cell (Reproduced with permission. Source: Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL (2003) Anti- TNF-α therapies: the next generation. *Nature Reviews Drug Discovery*. 2: 736–746. Nature Publishing Group)

recommended for patients with serious infections or sepsis and does not appear to result in the reactivation of tuberculosis.

2.23 Interleukin-7

IL-7 is a hematopoietic growth factor secreted by stromal cells (bone marrow and thymus), dendritic cells, keratinocytes, epithelial cells, neurons, and hepatocytes. It is not secreted by lymphocytes. IL-7 has three-dimensional structure that is complex. It induces the differentiation of pluripotent hematopoietic stem cells into lymphoid progenitor cells. The proliferation of B cells, T cells, and NK cells (lymphoid lineage) is induced by IL-7. It plays a role in the survival of various lymphocytes (T and NK cells), proliferation during some steps in B-cell maturation, and

development of homeostasis. T- and B-cell development requires IL-7, and it plays a crucial role in survival and proliferation of TH17 cells. In combination with hepatocyte growth factor, IL-7 constitutes a heterodimer, which is a pre-pro B-cell growth-stimulating factor. It is a cofactor during early T-cell development, where IL-7 is involved in the rearrangement of V(D)J of the T-cell β receptor. Intestinal epithelial and epithelial goblet cells secrete IL-7, and its local function is the regulation of intestinal mucosal lymphocytes. It is crucial for the survival of lymphoid cells.

IL-7 produces its physiological effects after binding to IL-7 receptors, which are composed of IL-7 receptor-α and a common γ chain. Binding of IL-7 to its receptors produces a series of signals, required for the development of T cells in the thymus and for their survival in the periphery. Administration of IL-7 in combination with anti-retroviral therapy inhibits local and systemic inflammation in HIV- or AIDS-infected patients who have incomplete T-lymphocyte reconstitution. IL-7 promotes the recovery of the immune cells after allogeneic stem cell transplantation. Clinical trials for IL-7 have been conducted for many forms of cancers, and it has been observed that there is a reduction in the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells and disruption in the homeostasis of both $CD4^+$ and $CD8^+$ T cells. There is development of neutralizing antibodies, which prevents dose-limiting cytotoxicity and no effects on cancer are observed.

2.24 Interleukin-32

IL-32 is a pro-inflammatory cytokine that is encoded by IL-32 gene. Expression of IL-32 is induced by T cells and NK cells. It augments the production of TNF-α from macrophages and IL-6 from T cells. IL-32 also enhances the synthesis and secretion of IL-8 and MIP-2/CXCL2. It supports the differentiation, but not activation, of the osteoclasts via its effects on MAPK/ERK pathway and the actin cytoskeleton.

2.25 Chemokines

Chemokines are a large family of small heparin-binding chemotactic cytokines released by many cell types. They are composed of four groups called CXC, CC, C, and CX3C. The designation and classification is based on the spacing of conserved cysteines and X is an amino acid. Many members constitute the CXC and CC groups, which is not the case for C and CX3C chemokines. Neutrophils and lymphocytes are the targets of CXC chemokines. The targets for CC chemokines are diverse, including basophils, dendritic cells, macrophages, and eosinophils. CXC family includes chemokines CXCL1-CXCL17, CC family includes CCL1-CCL28, C family includes XCL1-XCL2, and CX3C family includes only CX3CL1.

The early signals produced during innate immune responses are the main stimuli for the secretion of chemokines. A variety of chemokines are secreted by stimulus

resulting from viral infection, bacterial products (e.g., LPS), and pro-inflammatory cytokines including IL-1 and TNF-α. Consequently, some chemokines are pro-inflammatory in nature and are produced during an immune response to direct leukocytes to the site of injury/infection, whereas others are homeostatic in nature and control the migration of cells during routine tissue maintenance or development.

2.25.1 CXC Chemokines

Also termed as α-chemokines, they are composed of two N-terminal cysteines, separated by one amino acid designated with an "X" in the name. There are 17 different CXC chemokines and they are divided into two groups. One group has a specific motif of glutamic acid-leucine-arginine (ELR) right before the first cysteine of the CXC motif and is called ELR-positive. The other group does not have this motif and is called ELR-negative. ELR-positive CXC chemokines are specific for neutrophils and mediate their effects via CXCR1 and CXCR2 (CXC receptors 1 and 2). IL-8 is an example of ELR-positive chemokines, which directs the migration of neutrophils to the infected tissue. ELR-negative CXC chemokines, for example, CXCL13, are chemoattractant for lymphocytes.

2.25.2 CC Chemokines

Also termed as α-chemokines, they are composed of two adjacent cysteines near their amino terminus. There are at least 27 different CC chemokines of which CCL9 and CCL10 are the same. Most of the members of this group possess four cysteines (C4-CC) but a small number has six cysteines (C6-CC). CC chemokines regulate the migration of monocytes, dendritic cells, and NK cells. An important chemokine in this group is MCP-1 (monocyte chemoattractant protein-1 also called CCL2), which promotes the migration of monocytes from the blood stream to the tissue where they differentiate to become macrophages. Other CC chemokines include macrophage inflammatory proteins, MIP-1α (CCL3) and MIP-1β (CCL4), and RANTES (CCL5). The effects of CC chemokines are mediated via specific cell surface receptors: ten different types of these receptors (CCR1-CCR10) have been identified.

2.25.3 C Chemokines

Also termed as γ-chemokines, they are composed of only two cysteines: one on the N-terminal and the other a downstream cysteine. There are two chemokines in this group, lymphotactin-α (XCL1) and lymphotactin-β (XCL2). Their function is the attraction of T-cell precursors to the thymus.

2.25.4 CX3C Chemokines

Also termed as δ-chemokines, they are composed of three amino acids between the two cysteines. This subgroup has only one member, fractalkine (CX3CL-1). CXC3 chemokines is secreted as well as present on the cell surface and serves both as a chemoattractant and as an adhesion molecule.

2.25.5 Biological Role of Chemokines

The primary function of chemokines is to induce the migration of leukocytes. A signal directs these cells toward the chemokines. During immunologic surveillance, chemokines direct lymphocytes to the lymph nodes, which allow them to interact with the antigen-presenting cells and detect any invading pathogens. Such chemokines are called homeostatic chemokines and do not require a stimulus for their secretion. Some chemokines are pro-inflammatory in nature and require specific stimulus for their release. These stimuli include viral infection, bacterial products, as well as other chemical agents. Pro-inflammatory cytokines including IL-1 and TNF-α promote their release. These chemokines are chemoattractants for neutrophils, leukocytes, monocytes, and some effector cells, and they direct the migration of these leukocytes to the site of injury/infection. Some pro-inflammatory chemokines are also involved in wound healing similar to the pro-inflammatory cytokines. Chemokines are also capable of activating leukocytes to initiate an immune response and are involved in both innate and acquired immunity. Other chemokines play a role in development and are involved in angiogenesis and cell maturation.

2.25.6 Chemokine Receptors

Chemokine receptors are a family of G protein-coupled receptors which, contain seven transmembrane domains. Chemokine receptors are present on the cell surface membrane of leukocytes. As was the case for chemokines, these receptors are also divided into four subgroups: CCR is specific for CC chemokines, CXCR for CXC chemokines, XCR1 for C chemokines, and CX3CR1 for CX3C chemokines. CC chemokine receptor family has 11 members, CXC chemokine receptors family has 7 members, and both C chemokine receptor family and CX3C chemokine receptor family have 1 member each. The signal transduction is mediated via the standard G protein-dependent pathway.

2.26 Chemokines and Disease States

2.26.1 HIV Infection

HIV requires CD4 and either chemokine receptor CXCR4 or CCR5 to enter target cells. This allows the entry of HIV into $CD4^+$ T cells or macrophages, which eventually leads to the destruction of $CD4^+$ T cells and almost total inhibition of antiviral activity. The individuals that possess a nonfunctional variant of CCR5 and are homozygous for this gene remain uninfected despite multiple exposures to HIV. Clinical trials are under way to develop antagonists of these chemokine receptors as potential therapeutic agents for HIV infection and AIDS.

2.26.2 Diabetes with Insulin Resistance

Cytokines and chemokines have been implicated in insulin resistance. These cytokines, which may play a role include IL-6 and TNF-α. CCR2 are present on adipocytes, and activation of inflammatory genes by the interaction of CCR2 with the ligand CCL2 results in impairing the uptake of insulin-dependent glucose. Adipocytes also synthesize CCL2, resulting in the recruitment of macrophages. CCL3 may also be involved in insulin resistance.

2.26.3 Atherosclerosis

CCL2 is present in lipid-laden macrophages and the atherosclerotic plaques that are rich in these macrophages. The production of CCL2 in endothelial and smooth muscle cells is stimulated by minimally oxidized low density lipoproteins. As a consequence, CCL2 is involved in the recruitment of foam cells to the vessel wall. The patients who are homozygous for the polymorphism in the promoter of CCL2 appear to have a high risk for developing coronary artery disease as opposed to the patients who are heterozygous. CXCR2 and CX3CR1 are also implicated in cardiovascular disease.

2.26.4 Inflammatory Diseases

Chemokines are involved in a variety of inflammatory diseases including asthma, arthritis, psoriasis, and multiple sclerosis. Chemokine CC11 (eotaxin) and its receptors CCR3 are involved in the recruitment of eosinophils to lungs, contributing to the etiology/pathogenesis of allergic disease/asthma. Elevated levels of CCL2, CCL3, and CCL5 are found in the joints of patients with rheumatoid arthritis, which are involved in the migration of monocytes and T cells to the inflamed joint. In psoriasis, CCR4 is expressed on infiltrating effector T cells and cutaneous cells produce CCL17 and CCL22, which are ligands for CCR4. CXCR3 also plays a role in psoriasis. In multiple sclerosis, the levels of CXCL10 are elevated but there are lower levels of CCL2, and CXCR3 may also play

a role. Multiple sclerosis lesions contain many chemokines including CXCL10, CCL3, CCL4, and CCL8, where they may be predominantly involved in the migration of monocytes and macrophages from the peripheral blood into the tissue and lesions. The infiltrating monocytes express both CCR1 and CCR5.

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Chapter 3
Cytokine Receptors and Signaling

Abstract Cytokines exert their effects after binding to specific cell surface receptors, which are composed of polypeptide chains. After the binding of the ligand to its receptor, there is an initiation of the signal transduction pathways culminating in the synthesis of new mRNA and protein synthesis, in most cases. Some of these pathways are unique targets for therapeutic manipulations in disease state. This chapter focuses on five families of cytokine receptors, which constitute immunoglobulin superfamily receptors, class I cytokine receptor family (hematopoietin receptor family), class II cytokine receptor family (interferon receptor family), TNF receptor family, and chemokine receptor family. Furthermore, cytokine receptor-associated transcription factors are discussed. Lastly, JAK-STAT signaling pathway, inhibitors of Janus kinases, mitogen-activated protein kinases, mitogen-activated protein kinase inhibitors, protein inhibitors of activated STATs (PIAS), and suppressors of cytokine signaling (SOCS) are described. The inhibitors of Janus kinases, MAPK, and STAT5-associated ligands are clinically used to treat a variety of diseases.

Keywords TAK1 • TAB1 • TAB2 • TRAF6 • Immunoglobulin superfamily receptors • Class I cytokine receptor family • Class II receptor cytokine family • TNF receptor family • NF-κB • TRADD • gp39 (CD40-L) • CD27-L • CD30-L • JNK • ERK • p38 • Fas • CD40 • Chemokine receptor family • Signal transducer and activator of transcription (STAT) • STAT1 • STAT2 • STAT3 • STAT4 • STAT5 • INF-γ-activated sequence (GAS) • Imatinib • Nilotinib • Dasatinib • STAT6 • Janus kinases (JAK) • JAK-STAT signaling pathway • Tofacitinib • Ruxolitinib • Mitogen-activated protein kinases • Sorafenib • Protein inhibitors of activated STATs (PIAS) • Suppressors of cytokine signaling (SOCS)

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3.1 Introduction

The biologic effects of cytokines result after they interact with their highly specific cell surface receptors distributed on various tissues. The ligand–receptor interaction is quite limited to produce the biological responses since the number of cytokine receptors vary from as low as 10^2/cell to as high as 10^5/cell, and the range for their affinity is about 10^{10} M^{-1}. The cytokine receptors are generally composed of multiple polypeptide chains, all of which may interact for the successful transmission of the cytokine-induced signal. There are five families of cytokine receptors that include immunoglobulin superfamily receptors, class I cytokine receptor family (hematopoietin receptor family), class II cytokine receptor family (interferon receptor family), TNF receptor family, and chemokine receptor family (Fig. 3.1).

3.2 Immunoglobulin Superfamily Receptors

The immunoglobulin superfamily includes various cell surface receptors as well as soluble proteins. Their function includes recognition, binding, and adhesion among cells. These receptors share features with immunoglobulins as they all possess immunoglobulin domain or fold. This group contains receptors not only for cytokines but also for antigens and costimulatory molecules involved in the immune response. Although they share a similar immunoglobulin fold, there are differences in their distribution, composition, and biological functions.

One prominent member of this group, the IL-1R/TLR (interleukin-1 receptors/Toll-like receptors) superfamily of receptors, plays an essential role in innate immunity and inflammation. The autoimmune and inflammatory diseases are associated with these signaling pathways. Both IL-1 and IL-18 act through this family of receptors and as a result promote a wide range of proinflammatory mediators. IL-1R pathway is an ancient pathway of host defense. Another receptor homologous to IL-1R is Toll-like receptor (TLR) family, which is very important in innate immunity and include receptors for lipoteichoic acid (TLR2), LPS (TLR4), bacterial flagellin (TLR5), and CpG motifs in DNA (TLR9).

The downstream signaling molecules for this receptor family are shared by IL-1R, IL-18R, and TLR family, which include MyD88 (an adapter molecule), IRAKs (IL-RI-associated protein kinases), TAK1 (transforming growth factor-β-activated kinase), TAB1 and 2 (TAK1 and TAK2 binding protein), and TRAF6 (tumor necrosis factor receptor-associated factor 6). MyD88 is recruited after the binding of a ligand to IL-1R or TLR, resulting in the binding of IRAK4 and IRAK1, which are the IL-IR-associated kinases. In this process, the activation of IRAK4 hyper-phosphorylates IRAK-1 that allows the interaction of TRAF with the complex. This complex then binds to another complex, which includes TAK1, TAB1, and TAB2, resulting in the phosphorylation of TAB2 and TAK1 and migration of

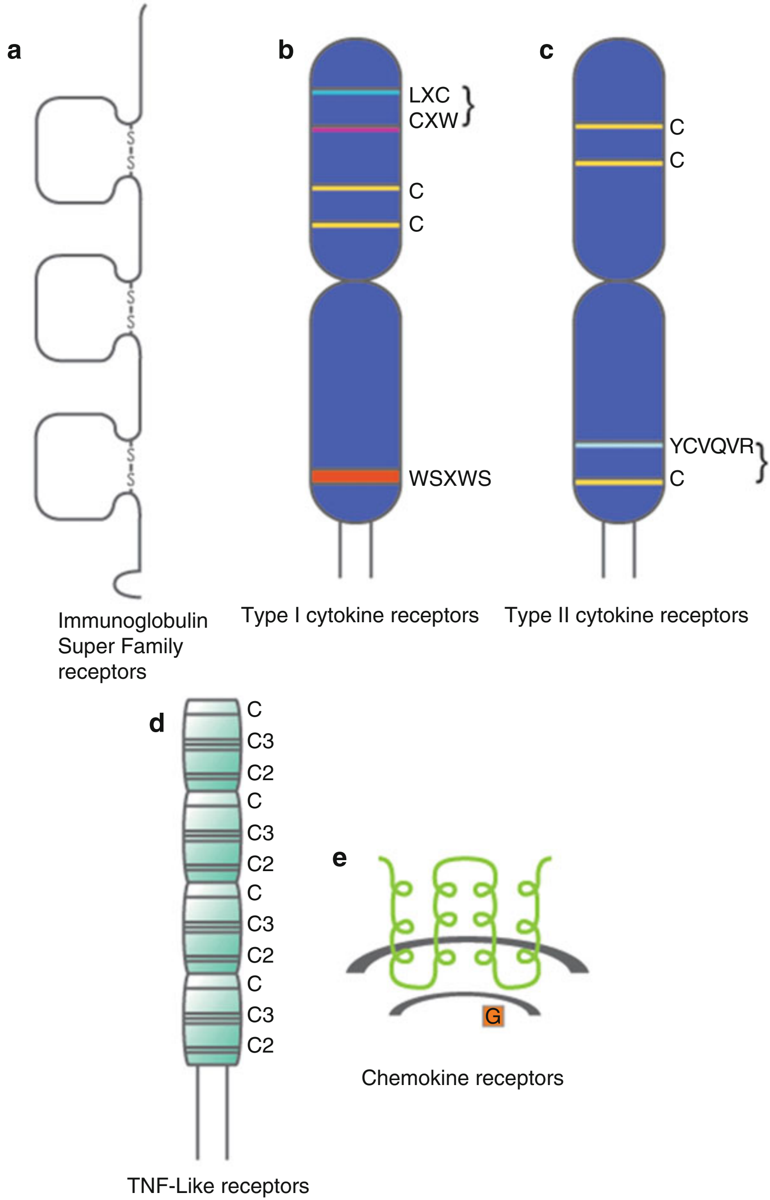

Fig. 3.1 Families of cytokine receptors: the cytokine receptors are classified into five major families which includes: (**a**) immunoglobulin superfamily receptors, (**b**) type I cytokine receptors, (**c**) type II cytokine receptors, (**d**) TNF-like receptors, and (**e**) chemokine receptors. The drawing illustrates their general biochemical structure

TAK1, TAB1, TAB2, and TRAF6 to the cytosol. The activation of TAK1 in the cytosol results in the phosphorylation of IKK, which degrades IκB and frees NF-κB. This signaling also results in the induction of MAPKs (mitogen-activated protein kinases) and JNK (Janus kinases) via activation of TAK1.

3.3 Class I Cytokine Receptor Family

Class I cytokine receptor family is the largest and most divergent family of cytokines, which include approximately 50 different interleukins, lymphokines, hematopoietins, growth hormones, and neuropoietins, and the majority of cytokine receptors belong to this family. These receptors are also known as hemopoietin receptors. The common amino acid motif (WSXWS) is located in the extracellular portion and is next to the cell membrane. Their members are made up of different chains; some interact with a cytokine/ligand, while others are responsible for signal transduction.

They are composed of 200 amino acids and contain two motifs; one is made up of four cysteine residues (CCCC) and the other is comprised of a conserved sequence of the amino acids tryptophan-serine-(any amino acid)-tryptophan-serine (WSXWS). The presence of two fibronectin type III modules gives it a "barrel-like" shape, and the cytokine-binding pocket is the trough formed in between two barrel-like modules. These receptors are heterodimers: one binds to the cytokine and the other is involved in downstream signal transduction. Class I cytokine receptors are composed of three subfamilies, and each subfamily member has identical signal transduction mechanisms through similar subunits. However, their cytokine-specific subunits are different, but redundancy and antagonistic properties of some cytokines result from similar signal transduction units.

The presence of at least two chains is the common characteristic of many of these receptors. Cytokines having two chain structures exhibit dual affinity although there are some exceptions. Type I cytokine receptors include IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7, IL-9R, IL-11R, IL-12R, IL-13R, IL-15R, IL-21R, IL-23R, IL-27R, erythropoietin receptors, G-CSFR, GM-CSFR, growth hormone receptors, prolactin receptors, leukemia inhibitory factor receptor, and oncostatin M receptors. The examples of receptors with two chain structures include sharing of γ–γ-subunit (CD132) of IL-2 with IL-4, IL-7, IL-9, IL-15, and IL-21; common β (CD131 or CDw131) chains of IL-3, IL-5, and GM-CSF; and common α-chain for IL-4 and IL-13. GP-130, which is a second chain required for IL-6-α activity, is also a trigger for several other cytokines. The gp230 receptor common ϒ chain (also called gp130, IL-6ST, IL6β or CD130) is shared among IL-6, IL-11, IL-12, IL-27, leukemia inhibitory factor receptor, and oncostatin M receptor. The binding of the cytokine to the double chain renders a dual affinity as is the case for IL-2, IL-3, IL-5, IL-7, and GM-CSF.

3.4 Class II Receptor Cytokine Family

The type II cytokine receptor family is composed of transmembrane proteins and is expressed on the membrane of some cells. Selected cytokines are their ligands and after binding a response is produced. They are also called interferon receptor family. These receptors have either one (IFN-γ) or two 200 amino acid domains; the domains contain cysteine in both carboxyterminal domains, and are also related to fibronectin type III domains, which are composed of 100 amino acids. Each fibronectin type III domain contains conserved Cys, Pro, and Trp residues, which are responsible for the formation of a folding pattern of seven-β strands. These β-strands are similar to the constant domain of the immunoglobulins. The class II receptor cytokine family lacks the WSXWS motif present in class I cytokine receptors. Type I INF-α/β and INF-γ are made up of two receptor chains, one for ligand binding and the other for signaling. This family includes receptors for IFN-α1, IFN-α2, INF-γ1, INF-γ2, IFN-κ (type I interferon), IFN-λ (IL-28/29), and molecules related to IL-10 (IL-19, IL-20, IL-22, IL-24, IL-26). The type I interferon receptors (IFNAR) have different components classified as IFNAR1 and IFNAR2. They have a number of cognate ligands including 13 IFN-α subtypes, β, ω, Є, κ, and others. The type I interferon receptors are from the type II INFγ (INFGR1 and INFGR2) and the type III INFs (INFLR and IL10Rβ).

Two ligand-binding interferon-γ receptor chains 1 in conjunction with two interferon-γ receptor chains 2 and associated signal-transducing proteins constitute functional INF-γ receptor (IFN-γR). This is a class of receptors that binds the agonist in the small angle of a V produced by the two Ig-like folds, which form the extracellular domain. The response to IFN-γ depends on the IFN-γR2 chain since there is an ample amount of the IFN-γR1 chain. The levels of the IFN-γR2 chain are strictly regulated depending on the activation and differentiation state of the cell despite their constitutive expression. The intrinsic kinase/phosphatase activity is not associated with either IFN-γR chain, and consequently they associate with signaling machinery for signal transduction. The binding motifs for the Janus tyrosine kinase 1 and signal transducer and activator of transcription (STAT1) are present in the intracellular domain of INFγR1. The JAK1 and STAT1 motifs are necessary for the phosphorylation and signal transduction of the receptors, as well as for the induction of the biological response. A noncontiguous binding motif for JAK2 kinase recruitment is present in the intracellular region of IFN-γR2 and participates in signal transduction.

IFN-λ is similar to type I IFNs in possessing antiviral and proliferation-inducing activities, but has distinct receptors, where CRF2 proteins are involved in the formation of heterodimers and mediating cytokine-induced signaling activity. IFN-λ is present in three forms, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). The ligand-binding chains for IFN-λ, IL-22, and IL-26 are different from IL-10, but all of them use IL-10 receptor 2 as a common second chain to form active receptor complexes. After a class II receptor ligand binds to its receptor, the receptor complex

is fully assembled and the JAKs, which are associated with the intracellular domains of class II receptors, are induced. This results in the phosphorylation of tyrosine residue in the receptor chain, which serves as docking sites for the transcription factors, STATs. Several different STATs are activated by these cytokines, which can then combine to other cytosolic proteins before migration to the nucleus.

3.5 Tumor Necrosis Factor Receptor Family

The TNF receptor family members include TNF-α, TNF-β, gp39 (CD40-L), CD27-L, CD30-L, and NGF (nerve growth factor). TNF-α has two types of receptors TNFR1 (CD120a, p55/p60) and TNFR2 (CD120b, p75/80), which have differential distribution. TNFR1 is ubiquitously distributed while TNFR2 is restricted to immune cells. TNFR1 is activated by both the membrane bound and soluble trimeric form of TNF, whereas TNFR2 responds to the membrane-bound form. These receptors possess four extracellular repeats of a cysteine-rich domain, which usually contain six conserved cysteine. Following binding to the ligand, trimers may also be formed by TNF receptors as their tips intrude between TNF monomers resulting in the dissociation of SODD (silencer of death domains) from the intracellular death domain. SODD is an inhibiting protein, and its dissociation allows binding of TRADD (TNF receptor death domain-associated protein), an adaptor protein, to the death domain. This serves as a platform for further association of transcription factors, which is followed by initiation of one of three possible mechanisms, activation of NF-κB, activation of MAPK pathways, or induction of death signaling. NF-κB is activated as a result of recruitment of TRAF2 (tumor necrosis factor receptor-associated factor-2) and RIP (receptor-interacting protein) by TRADD. Subsequently, protein kinase IKK is recruited by TRAF2, which is activated by RIP, a serine–threonine kinase. IKK also phosphorylates and degrades IKBα that is an inhibitory protein for NF-κB. This allows NF-κB to migrate to the nucleus and direct gene expression of a number of inflammatory proteins and inhibit apoptosis.

TNF also activates MAP kinases including stress-related JNK and P38-MAPK, but extracellular signal-related kinases (ERKs) are not significantly activated. JNK-inducing upstream kinases are induced by TRAF2 resulting in the activation and translocation of JNK into the nucleus where it activates other transcription factors resulting in cell proliferation and differentiation.

Lastly, TNF-R1 is involved in apoptosis. However, this is not a major function as opposed to their vast role in inflammatory processes. This is due to interference of its anti-apoptotic effects by NF-κB and Fas, another family member that exhibits more potent apoptotic effects. The apoptotic effects result from the binding of TRDD to Fas-associated death domain protein (FADD), which is followed by the recruitment of cysteine protease caspase-8. High concentrations of caspase-8 induce cell apoptosis after its auto-proteolytic activation and cleaving of effector caspase. The Fas antigen member of TNF receptor gene family has been called the "death gene," and Fas ligands related to TNF and CD40L trigger apoptosis. CD40 is a

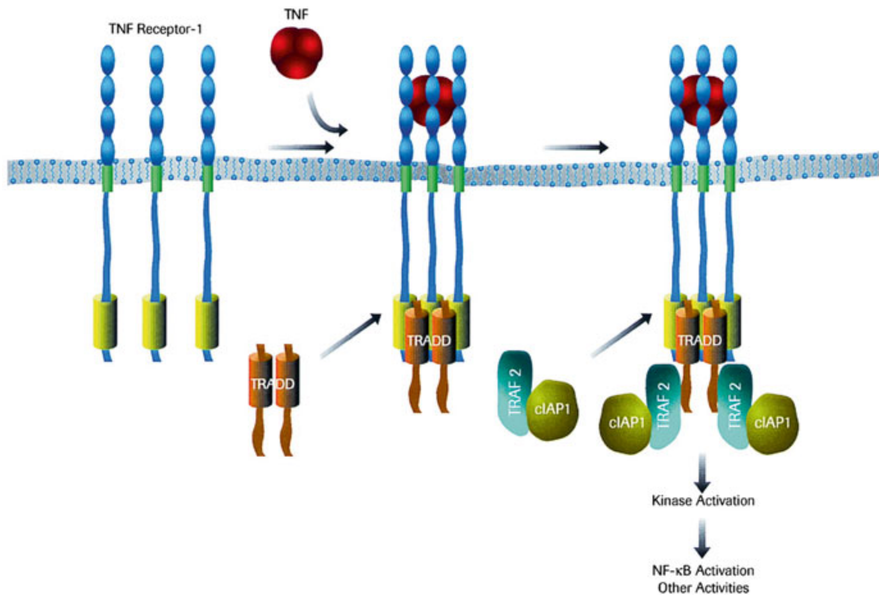

Fig. 3.2 An illustration of TNFR1 signaling complex (Reproduced with permission. Source: Hong-Bing Shu, Masahiro Takeuchi, and David V. Goeddel (1996) The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. 93:13973–13978. Copyright (1996) National Academy of Sciences, U.S.A.)

natural ligand protein present on activated T cells and is critical for T cell-dependent B-cell activation. The regulation of apoptosis is also the function of the other members of this family, including nerve growth factor receptors, CD27 and CD30 (Fig. 3.2).

3.6 Chemokine Receptor Family

The chemokine receptor family is also termed as G-protein-coupled receptor superfamily of proinflammatory cytokines. It includes IL-8, MIP-α, f-met-leu-phe, and C5a as ligands. These receptors have seven predicted transmembrane domains and use G-proteins as signal transducers. G-proteins couple this family of receptors, which activate adenylate cyclase and phospholipase C. These are structurally related receptors and are divided into four families: CXCR binds CXC chemokines, CCR binds CC chemokines, CX3CR1 only binds CS3C, and XCR1 binds two chemokines, XCL1 and XCL2. The ligand-binding specificity of a chemokine receptor is dependent on the N-terminal portion of chemokine receptors. All chemokine receptors contain approximately 350 amino acids with a short acidic N-terminal; seven helical transmembrane domains, each of which contains three intracellular and

extracellular loops; and a serine- and threonine-containing intracellular C-terminus. Signal transduction of chemokine receptors is achieved via G-proteins, which after activation induce phospholipase C, resulting in the production of inositol triphosphate and diacylglycerol. A number of signaling pathways are activated as a result of production of these two second messengers.

Chemokine receptors are expressed on the surface of certain cells and interact with chemokines. Nineteen different chemokine receptors have been identified. Each possesses seven-transmembrane (7TM) structure. After the chemokine ligand binds to its receptor, there is intracellular flux of calcium (Ca^{2+}) ions causing calcium-dependent signaling. This results in chemotaxis, which causes the migration of cell to a specific site within the organism. Signal transduction by chemokine receptors depends on the neighboring G-proteins. After binding of the chemokine ligand to its receptor, chemokine receptor associates with G-protein that permits the exchange of GDP for GTP, along with dissociation of other G-protein subunits. The subunit Gβ activates phospholipase C, which is present within the cell membrane. PLC cleaves phosphatidylinositol (4, 5) – bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 causes the release of calcium from intracellular stores and DAG induces protein kinase C (PKC). As a consequence, many signal transduction pathways are activated, which regulate the cellular response. As an example, binding of chemokine CXCL8 (IL-8) to its receptors, CXCR1 or CXCR2, causes an increase in intracellular Ca^{2+} levels, which induces phospholipase D and the signaling pathway culminating in the activation of mitogen-activated protein kinase (MAPK) pathway. The desensitization is achieved by activation of protein tyrosine kinase (PTK) via G-protein subunit Gα. This is the result of phosphorylation of threonine and serine residues in the chemokine receptors. The MAP kinase pathway regulates chemotaxis, release of free radical oxygen, and the effector function of integrins.

CXC chemokine receptors include seven members, CC chemokine receptors have 11 members, C chemokine receptors consist of one member, and CX_3C chemokine receptors possess one member (Fig. 3.3).

3.7 Cytokine Receptor-Associated Transcription Factors

3.7.1 Signal Transducer and Activator of Transcription (STAT)

A number of transcription factors are involved in downstream signaling pathways in response to cytokines, hormones, and growth factors. One such prominent family of the transcription factors is that of the signal transducer and activator of transcription. There are seven members of STAT family, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, which are made up of 750–850 amino acids and have molecular weight ranging from 90 to 115 kDA. They are constitutively present

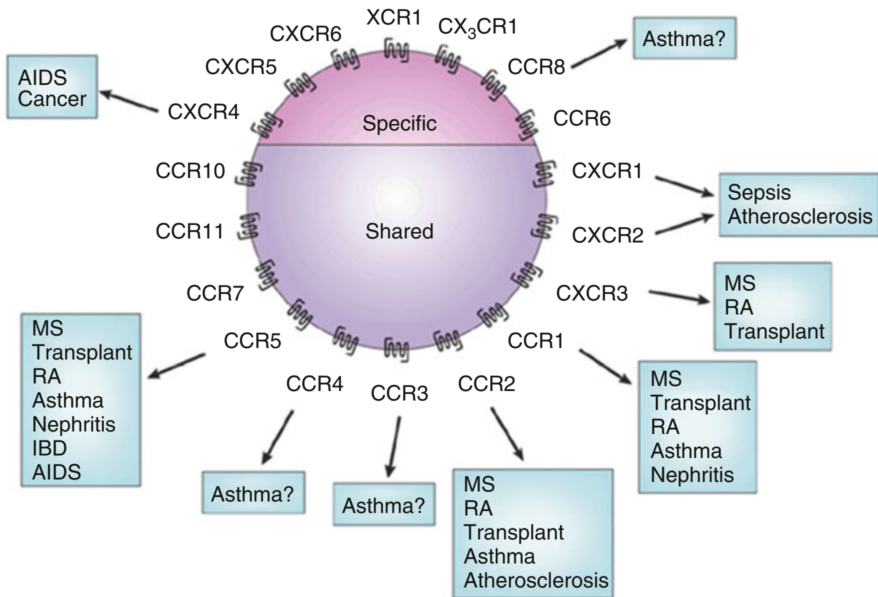

Fig. 3.3 Chemokine receptor family and their association with disease states (Reproduced with permission. Source: Proudfoot AE, (2002) Chemokine receptors: multifaceted therapeutic targets. *Nature Review Immunology* 2:106–115. Nature Publishing Group)

in an inactive form in the cytoplasm and are activated by their respective agonists including cytokines after they bind to their receptors. After their activation, STATs participate in gene activation and expression, where they play a dual role. In the cytoplasm they bind to the receptor-associated kinases and consequently relay the message from the cell surface to the nucleus, and after translocating to the nucleus and binding to DNA, they activate transcription. The association of STAT proteins with human disease is shown in Table 3.1.

3.7.1.1 STAT1

STAT1 is activated by a variety of ligands including cytokines, IFN-α, INF-γ, IL-4, IL-5, IL-13, growth factors, and hormones. It has four conserved domains: N-terminal, C-terminal, SH2 domain, and DNA-binding domain, which are typical for all STAT family members. The SH2 domain is distinct for each STAT, and a stable SH2-phosphotyrosine bond is formed after the SH2 domain binds to its specific phosphotyrosine domain. This SH2–pTyr bond is responsible for STAT activation by

Table 3.1 STAT proteins and human disease

STAT	Ligand	STAT deficiency-associated human disease
STAT1	IFN	Alzheimer's disease, cancer, celiac disease, ischemic heart disease, IBD, rheumatoid arthritis
STAT2	IFN	Cancer, IBD
STAT3	IL-2, IL-6, IL-10	Cancer, IBD, multiple sclerosis
STAT4	IL-12	COPD, rheumatoid arthritis
STAT5A	PRL	Cancer, diabetes, ischemic heart disease
STAT5B	GH	Growth retardation
STAT6	IL-4, IL-13	Allergic disease/asthma, cancer, ischemic heart disease

recruiting STAT to its specific cytokine receptor, binding to JAK–STAT, and dimerization of STAT–STAT. The N-terminal domain has a tyrosine residue, which is phosphorylated by activated JAKs, resulting in the dimerization and activation of STAT. The DNA-binding domain is connected to the SH2 domain and to the DNA sequence resulting in gene transcription. The C-terminal is important for transcriptional activity. STAT1 is activated by JAK as it phosphorylates the transcription factor assisting in its recruitment to the receptor, dimerization, and nuclear translocation. This results in the formation of a physical link between the STAT and the associated receptor. In response to either interferon-α or interferon-β, STAT1 forms homodimers or heterodimers with STAT3 that bind to the Interferon-Gamma-Activate Sequence (GAS) promoter element. STAT1 forms a heterodimer with STAT2, which binds to interferon-stimulated response element (ISRE). The binding of promoter element results in increased expression of interferon-stimulated genes (ISG).

Most of the biological actions of INF-γ are STAT1 dependent, although IFN-γ receptors activate additional signaling pathways and can regulate gene expression without the involvement of STAT1. Binding of IFN-γ to its receptors results in the phosphorylation and oligomerization of the receptors. As opposed to many growth factors that possess intrinsic kinase activity, cytokine receptors require JAKs for phosphorylation. The transphosphorylation of JAK at the tyrosine residues results in the recruitment of STAT1 to the cytokine receptor where the binding of the SH2 domain of the receptor to the pTyr residues of the JAKs takes place. The formation of either homo (STAT1/STAT1) or hetero (STAT1/STAT2) dimers occurs after the activated JAKs phosphorylate STAT1. These dimers migrate to the nucleus to bind to the target sequences on the DNA, inducing the transcription of STAT1-dependent genes. These genes include STAT1, ICAM1, RANTES, and interferon regulatory factor (IRF1). IFN-stimulated gene factor (ISF3γ) is required for the binding of STAT1/STAT2 heterodimer to the IFN-stimulated response element (ISRE) on the DNA. The binding of other cytokines, which utilize STAT1 as a transcription factor, to their receptors results in the same pathway.

In addition to JAKs, STAT1 is also directly phosphorylated by protein kinase C. This process is mediated by inositol triphosphate, Ca^{2+} release, formation of Ca^{2+}-calmodulin complex, and release of calcineurin. Calcineurin dephosphorylates NF-AT resulting in its translocation to the nucleus and subsequent activation of STAT1, in addition to other genes.

STAT1 plays an important role in biological function, since in its absence tissues are no longer able to respond to IFN-γ, becoming susceptible to viral infections. STAT1 is also a tumor suppressor due to its ability to promote growth arrest and apoptosis. It mediates the expression of inhibitors of cyclin-dependent kinases. Furthermore, it suppresses genes necessary for entry into the cell cycle. STAT1 mediates the effects of biological therapies of leukemia as well as the induced differentiation of leukemia cells. It is also a key negative regulator of angiogenesis. In lung and prostate cancers, there is a loss in the ability of IFN-γ to activate STAT1. Increased survival of the newly diagnosed breast cancer patients and a decrease in relapse are associated with enhanced phosphorylation and DNA binding by STAT1. The involvement of STAT1 in asthma is evident from the expression of ICAM1 and RANTES after its activation since they are involved in the recruitment of inflammatory cells at the site of inflammation. STAT1 is also associated with the inflammatory process in Alzheimer's disease.

3.7.1.2 STAT2

STAT2, a critical transcription factor for type I interferons, is present primarily in cytoplasm and is tyrosine phosphorylated following the binding of INFα to its cell surface receptors. After tyrosine phosphorylation, STAT2 migrates to the nucleus and binds to DNA along with STAT1 and IRF9. α/β interferons mediate their antiviral effects via STAT1 and STAT2 but not via STAT3. STAT2 is critical for IFN responsiveness in target cells. After recognition of interferon by its receptors, STAT2 causes the formation of a complex with STAT1 and IFN regulatory factor family protein p48 (ISGF3G). STAT2 serves as a transcativator while lacking the ability for direct DNA binding. It attracts transcription adaptor P300/CBP (EP300/CREBBP) and is involved in inhibiting IFN-α response to adenoviruses. It interacts with CREB-binding protein, MED13, STST1, IFNAR1, IFNAR1, ISGF3G, and SMARCA4. The antiviral and growth inhibitory effects of IFN are not observed in the absence of STAT2. For the IFN-stimulated gene factor 3 (ISGF3), a potent transactivational domain is provided by STAT2. STAT2/1 and STAT2/3 are ISGF3 independent and bind a γ-activated sequence-like (GAS) element.

3.7.1.3 STAT3

STAT3 was originally identified as a DNA-binding activity from IL-6-stimulated hepatocytes that selectively interacted with an enhancer element in the promoter of acute phase gene, known as the acute phase response element. Further characterization revealed that STAT3 was closely related to STAT1 and was activated by IL-6 type cytokine family, which signals through gp130 and/or related receptors. Many unrelated agonists such as oncogenes, growth factors, and IFNs may also activate STAT3.

STAT3 exists in two isoforms, a long form (STAT3α) and a short form (STAT3β). STAT3α possesses a STAT family DNA-binding domain, a major serine phosphorylation site at S727, a major tyrosine phosphorylation site at Υ705, and an SH2

domain. It is dimerized as a result of pTyr-SH2 interactions after it is phosphorylated and it could also heterodimerize with STAT1, and after phosphorylation STAT3 migrates to the nucleus. IL-6 and a number of other cytokines and growth factors can cause the induction of STAT3 through their respective receptors.

IL-10R produces a strong anti-inflammatory response via STAT3, which serves as an antagonist to proinflammatory signals that are activated during the innate immune response. However, anti-inflammatory response is not unique to the IL-10R-dependent signal transduction, but can be produced by a number of cytokines, which activate STAT3. STAT3β (short form) lacks C-terminal domain that is induced by phosphorylation of Serine 727 and is the domain for transcriptional activation. STAT3 is also known as an acute phase response factor and has ubiquitous distribution. Many forms of cancers including breast cancer, head and neck cancer, prostate cancer, and glioblastoma exhibit an enhanced STAT3 activity. Induction of STAT3 is also associated with inflammatory and autoimmune diseases such as acute lung injury, pulmonary fibrosis, and Crohn's disease.

3.7.1.4 STAT4

STAT4 is very important in mediating proinflammatory immune response, and based on its restrictive distribution of mRNA expression in myeloid and lymphoid tissues, it is evident that STAT4 is a distinct transcription factor. Cytoplasmic STAT4 exists in a latent form and its homodimers are formed after induction by cytokines via their specific receptors. Similar to other STATS, this is followed by migration of the dimer to the nucleus, binding to its niche in DNA, and the subsequent gene expression.

STAT4 is responsible for IL-12-mediated functions and for the development of TH1 cells that secrete IFN-γ from naïve $CD4^{+}$ T cells. After IL-12 induces its receptors, STAT4 binds to IL-18γ1 promoter, which results in an increase in acetylated histones H3 and H4 transiently. The transient hyperacetylation induced by STAT4 inhibits DNA methyltransferase recruitment and the consequent suppression of the IL-18γ1 locus.

STAT4 is induced not only by IL-12 but also by IFN-α and IL-23. IL-12 and IL-23 are produced in response to a variety of pathogenic organisms and regulate innate and acquired immune responses. IL-12 binds to IL-12β1 and IL-12β2 receptors. A subunit called P40 is shared by IL-12 and IL-23. IL-12 and IL-23 activate the JAKs, JAK2 and TYK2, STAT4, and other STATs.

The transduction activity of STAT4 involves several cell types and at different stages in the immune response. Its role in innate immunity is the production of IFN-γ and chemokines by macrophages, dendritic cells, and NK cells. These early acting cells may also be involved in STAT-dependent tissue destruction. A lack of STAT4 shifts the TH1/TH2 balance to TH2 cells and results in a decrease in the associated inflammatory response. A deficiency of STAT4 results in augmented inflammatory responses and susceptibility to a variety of infections.

STAT4 is required not only for most of the biological responses produced by IL-12, which includes IFN-γ production, but is also essential for the normal differ-

entiation of TH1 cell and for the expression of TH1-specific genes. These genes include IL-12 Rβ2, IL-18Rα, LT-α, and selectins. STAT4 deficiency protects from T cell-mediated autoimmunity but not from predominantly antibody-mediated autoimmune diseases. Cytokines activating STAT4 produce biological responses that render protection against microbes such as *Mycobacteria tuberculosis*, *Toxoplasma gondii*, *Listeria monocytogenes*, *Trypanosoma cruzi*, *Schistosoma mansoni*, *Leishmania major*, and others. However, unregulated stimulation of STAT4 produces inflammatory diseases such as arthritis, myocarditis, colitis, experimental autoimmune encephalomyelitis, diabetes, and others.

STAT4 suppresses TH2 cytokine production and supports TH1 cytokine production. STAT4 activation in $CD4^+$ T lymphocytes results in the differentiation of TH1 cells, which inhibit the development of IL-4-secreting TH_2 cells. A role of histamine type I receptors in histamine-mediated upregulation of STAT4 phosphorylation has been proposed. Histamine shifts TH1/TH2 cytokine balance from TH1 to TH2 cytokines, which may contribute to the etiology and pathogenesis of allergic disease/asthma.

3.7.1.5 STAT5

Among the seven members of the STAT family, STAT5 is the most ubiquitously activated transcription factor. It exists in two iso-forms, STAT5A and STAT5B. STAT5 is activated by a number of cytokines and growth factors including IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-15, GM-CSF, thrombopoietin, erythropoietin, prolactin, and growth hormone. Its predominant role is the regulation of mast cells and IL-3. Stem cell factor (SCF) activates STAT5 to regulate the development of mast cells, and both STAT5A and B are involved in this function. Cytokine receptor-induced JAKs cause dimerization, migration to the nucleus, and DNA binding after tyrosine phosphorylation of STAT5. Its regulation of expression of cytokine target genes, takes place after STAT5 binds to INF-γ-activated sequence (GAS) motifs. This is followed by STAT5 contacting coactivators and parts of the transcription apparatus, resulting in transcriptional activation. Serine/threonine kinase Pim-1, a target gene for STAT5, inhibits STAT5 activity by cooperating with SOCS1 and SOCS3.

STAT5 is required for the proliferation of T cells and B cells, as well as the self-renewal of hematopoietic stem cells. Tumor development may result from aberrant regulation of STAT5 activity. STAT5 is constitutively activated via cytokines or growth factor receptors and/or their associated tyrosine kinases and assists in the survival of malignant cells in a variety of cancers, including leukemia and head, neck, prostate, and breast cancers. This could be the result of either mutations or abnormalities in cell signaling. As a consequence, there is poor or an absence of regulation for the induction of transcription of STAT5-dependent genes. This enhances continuous expression of these genes. The example includes mutations in anti-apoptotic genes, preventing cell death. Their continuous presence results in the development of cancer. Signaling activators such as PI3-kinase and Ras help STAT5, and apoptosis can be induced in these tumors by inhibiting STAT5, which may have therapeutic implications for treating certain forms of STAT5-sensitive tumors.

Targeting STAT5 for the treatment of cancer has included approaches to both directly and indirectly suppress constitutively phosphorylated STAT5 activity. The purpose of indirect targeting is to inhibit kinases associated with STAT5. One example is imatinib, which inhibits BCR/ABL (Bcr-Abl tyrosine kinase). For cell survival, cell signaling cascade is needed to keep them alive. Phosphatase group serves as an off and on switch for some of these proteins. A tyrosine kinase enzyme adds this phosphatase group. This tyrosine kinase enzyme needs to be turned on and off under normal circumstances. However, in some patients with Ph-positive CML, one tyrosine enzyme, BCR-ABL (the Abelson proto-oncogene) is stuck in an on position and continues to add phosphate groups. Imatinib is an inhibitor of this continued phosphorylation process. This enzyme is only present in cancer cells and the drug is selective for those cells and as a result is considered as targeted therapy. Imatinib is used to treat many forms of cancers, including chronic myelogenous leukemia (CML), gastrointestinal stromal tumors, myelodysplasia or myeloproliferative diseases, metastatic dermatofibrosarcoma protuberans, and aggressive systemic mastocytosis. It is absorbed rapidly when orally administered, and 98 % of the dose reaches the bloodstream. Imatinib is metabolized in the liver by cytochrome p450 system. As a consequence, the drugs interfering with cytochrome p450 system will interfere with its effects. The possible side effects of imatinib include headache, cough, nausea, diarrhea, anxiety, joint pain, stomach pain, tiredness, muscle pain, taste changes, sweating, weakness, and sleep disorder.

Nilotinib (Tasigna), another tyrosine kinase inhibitor, is used to treat imatinib-resistant chronic myelogenous leukemia. In a study after 5 months of treatment with nilotinib, 92 % of the patients resistant to imatinib exhibited normal leukocyte numbers. However, nilotinib may cause possible heart complications. Other side effects include fatigue, nausea, vomiting, diarrhea, headache, flu-like symptoms, rashes, and muscle pain. This drug is also under investigation to treat Alzheimer's disease, dementia, ALS, Parkinson's disease, and Huntington's disease.

Dasatinib (Sprycel) is also a Bcr-Abl tyrosine kinase inhibitor based on indirect STAT targeting and is also used to treat first-line chronic myelogenous leukemia patients (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia. The side effects include peripheral edema, diarrhea, headache, nausea, vomiting, peeling skin, rashes, weakness, joint pain, loss of appetite, stomach pain, myelosuppression, neutropenia, and pulmonary hypertension. The drug is also being evaluated to treat other forms of cancers including advanced prostate cancer.

3.7.1.6 STAT6

STAT6, an important transcription factor, mediates IL-4 and IL-13 receptor-induced signals. It is composed of 850 amino acids and its gene is present on the 12q12-q24 region of the human chromosome. Like other STATs, STAT6 has four functional domains: N-terminal domain, C-terminal domain, a DNA-binding domain, and a conserved Src homology 2 (SH2) domain. STAT6 plays an important role in TH2 differentiation and TH2 responses are not present in STAT6-deficient mice.

The binding of IL-4 or IL-13 to their respective receptors activates JAK–STAT6. IL-4 and IL-13 share the IL-4Rα chain of the IL-4 receptor. After IL-13 binds to IL-13Rα1, it recruits IL-4Rα, resulting in a high-affinity complex for ligand binding and signaling. This results in the activation of JAK1 and JAK3 tyrosine kinases followed by tyrosine phosphorylation. These steps create the docking site for STAT6, which is then phosphorylated, homodimerized, and migrated to the nucleus for gene expression. In addition to playing a critical role in TH2 differentiation, which is critical role in the pathogenesis of allergic disease/asthma, STAT6 also promotes eotaxin secretion, a crucial factor for the eosinophilic infiltration in the airways.

3.8 Janus Kinases

Four mammalian Janus kinase (JK) family members have been identified: JAK1, JAK2, JAK3, and Tyk2 with molecular weights ranging from 120 to 140 kDa. JAK1, JAK2, and Tyk2 are expressed ubiquitously, whereas JAK3 is present only on hematopoietic cells. JAKs are activated by cytokines that share a common α-helical structure and use additional receptors for signal transduction. For ligand binding, the cytokine receptors are made up of polypeptides with a single transmembrane domain as well as common extracellular motifs. There is no intrinsic catalytic activity present in the cytokine receptors; as a consequence, JAKs are required, which are present in the cytoplasmic region of the receptor and with their association the extracellular signal is transmitted intracellularly. The JAKs' association with the cytokine receptors requires motifs, which are called box 1 and box 2. Binding of the agonist to its receptors within minutes activates JAKs resulting in the dimerization or oligomerization of the subunits of the receptors, and pre-existing dimers undergo conformational changes after agonist binding to the cytokine receptors. If the ligand binds to homodimeric receptors, only JAK2 is activated; however, binding to heterodimeric receptors activates a combination of JAKs. Following activation, the receptor subunits are phosphorylated as well as other substrates including STATs (Fig. 3.4).

JAKs are composed of an amino terminal region (N), catalytically inactive kinase-like (KL) domain, and a tyrosine kinase domain (TK). The family is distinguishable on the basis of the presence of an additional kinase-related domain. There are seven regions of homology in JAKs called JH1, JH2, JH3, JH4, JH5, JH6, and JH7, which are present from carboxy to the amino terminus. JH1 corresponds to TK domains, JH2 to KL, and JH3–JH7 to N regions. The N region of the JAKs is composed of 550 amino acids and this region is responsible for binding to the cytokine receptors. The cytokine receptor binding specificity is homed in JH7 and part of JH6, which is comprised of the first 200 residues of the JAKs. KL domain does not possess motifs required for the catalytic activity but shares similarities with TK. However, KL domain can play a role, which is not dependent on TK domain and is considered to be a negative regulatory domain. TK domain is responsible for the phosphorylation of the cytokine receptors that is accomplished by the phosphorylation of tyrosine

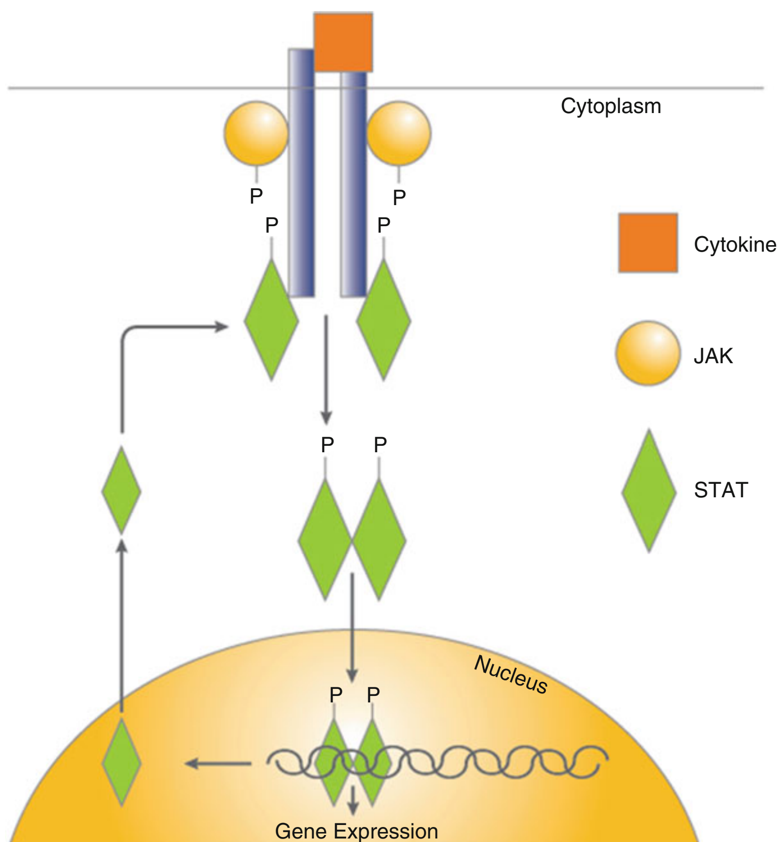

Fig. 3.4 The JAK/STAT signaling pathway; after cytokine binds to its receptors the associated Janus kinase (JAK) is induced. This results in the phosphorylation of the receptor's cytoplasmic domain. STAT is recruited after the phosphorylation of the receptor's cytoplasmic domain which after phosphorylation dimerizes and migrates into the nucleus. In the nucleus STAT binds to its niche in the DNA and induces gene expression

residues. These tyrosine residues are present in a loop in the TK domain, and the tyrosines that are phosphorylated are members of the JAK family.

3.9 The Janus Family Tyrosine Kinases–Signal Transducers and Activator of Transcription Signaling Pathway

The Janus family tyrosine kinases–signal transducers and activators of transcription signaling pathway are the best-understood cytokine receptor signaling cascade. Four Janus kinases (JAK1, JAK2, JAK3, and TyK2) and seven signal transducers and activators of transcription (STAT) factors mediate signal transduction of almost

Table 3.2 Some transcription factors which interact with JAK

JAK	Transcription factors
JAK2	STAT3
JAK1, JAK2	STAT5
JAK3, TYK2	
JAK2, JAK2, TYK2	Raf-1
JAK1, JAK2	Grb-2
JAK1, JAK2, JAK2, TYK2	SOCS1
JAK1	PI3K
JAK1	CPLA2
JAK1, TYK2	SHP1
JAK1, JAK2	SHP2
TYK2	Crk-L

40 cytokine receptors. The binding of cytokine to its receptors induces the associated JAK, resulting in the phosphorylation of the receptor's cytoplasmic domain. This phosphorylation permits the recruitment of STAT, which after phosphorylation dimerizes and migrates to the nucleus, where it binds to its niche in the DNA and induces gene expression. The JAK binding sites are present close to the cell membrane where association of the JAKs with the cytoplasmic domains of cytoplasmic receptors takes place. After cytokine binds to its receptor, close proximity of JAK permits its induction. The binding sites for the Src homology 2 (SH2) domains of the STATs are created as a result of phosphorylation of cytokine receptor by the JAK. The migration to the nucleus follows tyrosine and occasionally serine phosphorylation of STAT by the JAKs as well as other kinases. Multiple cytokine receptors may be present on a single cell that may coordinate all the signals coming from multiple receptors. One JAK or a combination of JAKs is used by a class of receptors, for example, some receptors use only JAK1, some cytokines involved in hematopoiesis and proliferation use JAK2, and additional cytokines which have common γ-chain receptors use JAK1 and JAK3. The only exception is TYK2, which is involved in signaling of many different classes of receptors. A summary of some transcription factors that interact with JAK and their stimulatory cytokines signals is shown in Tables 3.2 and 3.3.

Activated cytokine receptors in different cell types cause both cell type-specific and core transcription. The examples include the expression of similar cohort of genes in any cell type induced by IFN-γ via STAT1, which overlaps with the gene expression caused by IFN-αβ. IFN-αβ signaling also utilizes STAT1, in association with STAT2 and IRF9. These genes are called the "IFN signature" and are reflective of the activity of STAT1. An example of cell type-specific pathway is IL-4- or IL-13-induced STAT6 pathway. The mechanism of activation of genes in T cells by IL-4 is more distinct than in macrophages or other cell types, which also applies to IL-13-mediated signaling. This suggests that specific gene expression is regulated by STATs based on their accessibility and with the involvement of other cofactors.

The importance of STATs in cytokine receptor-mediated signaling has been further elucidated by using STAT knockout mice. These studies have demonstrated that

Table 3.3 JAK/STAT signal transduction

Cytokines	JAK kinase	STATs
IL-2, IL-7, IL-9	JAK1, JAK3	STAT3, STAT5
IL-3, IL-5, GM-CSF	JAK2	STAT5
IL-4	JAK1, JAK3, or JAK2	STAT6
IL-6, IL-11, G-CSF	JAK1, JAK2, TYK2	STAT1, STAT3, STAT5
IL-10	JAK1, TYK2	STAT1, STAT3
IL-12	JAK2, TYK2	STAT4
IL-13	JAK1, JAK2, TYK2	STAT6
IL-15	JAK1, JAK3	STAT3, STAT5
IFN-α, IFN-β	JAK1, TYK2	STAT1, STAT2, STAT3
IFN-γ	JAK1, JAK2	STAT1

the genes regulated by IFN-γ that provide immunity against pathogens are dependent on STAT1. Similarly, the genes, which are required for hematopoietic survival require STAT5A and STAT5B.

The signaling from receptors using the same JAK–STAT pathway in the same cell is different. For example, JAK1-STAT3 pathway is activated in macrophages when they bind to either IL-6 or IL-10, but the downstream signaling pathways in macrophages for IL-6 and IL-10 are different irrespective of the fact that both utilize JAK1-STAT3 pathway. IL-10, a negative regulator of inflammation, utilizes STAT3 and indirectly targets a few STAT3-regulated genes. However, IL-6, despite using JAK1-STAT3 pathway, does not induce the anti-inflammatory response. This has been attributed to the ability of the negative regulator of JAK–STAT activity SOC3 that controls IL-6R-mediated responses, since other cytokines that are SOC3 independent and utilize JAK1/STAT3 pathway can produce anti-inflammatory response.

3.9.1 The JAK/Cytokine Receptor Interaction

Seven JAK homology (JH) domains have been identified according to sequence similarities in JAK family members, which partially match the domain structure of JAK. The classical kinase domain is the JH1 domain at the C-terminus, preceded by the JH2 domain on the N-terminal, which is also called pseudo-kinase domain, and does not have crucial residues for catalytic activity and for binding of nucleotides, although it has a kinase domain and modulates the kinase activity. The cytokine receptor binding is accomplished by JH3-JH7 regions, which are the N-terminal half of the JAKs. There is remarkable similarity of sequence between a segment of the N-terminal region of the JAKs and Ferm (four point-1, ezrin, radixin, and moesin) domains. The Ferm domains are composed of three subdomains: F_1, F_2, and F_3. The F_1 subdomain has an ubiquitin-like β-grasp fold, the F_2 subdomain has an acyl-CoA-binding-protein-like fold, and the F_3 subdomain shares phosphotyrosine-binding or pleckstrin homology domains. Ferm domains play an important role in the association of JAKs with cytokine receptors.

The proximal region of the cytokine receptors' membrane is the binding site for JAKs. The receptors have diverse sequences except sequence homology in box 1 and box 2 regions, which are short stretches where the box 1 region is made up of eight amino acids that are proline rich, and the box 2 region is an aggregate of hydrophobic amino acid residues. Areas C-terminal of box 2 in some cytokine receptors participates in binding to JAK and its activation. The signaling involves the interface interaction between large segments of the receptor box 1/box 2 region and the N-terminal region of the JAKs resulting in correct alignment of JAKs; thus, agonist binding to the receptor causes dimerization and reconfiguration of the receptor and activation of both the JAKs and the receptors.

JAK association requires structural integrity of α structure. Two to three proline residues are present in the box 1 motif's C-terminal part. There is restructuring of certain receptor residues resulting from cytokine receptor–JAK interaction causing defined interaction, which is termed as "induced fit-like" interaction, causing defined interaction interfaces. The reorganization of the JAK–cytokine receptor binding interface is also possible as a result of activation of JAKs. A high-affinity association of JAK with cytokine receptors results in its recruitment to the membrane where there is no exchange of JAK molecules between different receptors.

In addition to serving as second messengers for cytokines, JAKs may also regulate cell surface expression of certain cytokine receptors. For example, the co-expression of JAK1, JAK2, or TYK2 augments OSMR (oncostatin M receptor) expression. The expression of EpoR (erythropoietin receptor) requires JAK2 and also assists in its folding process in the endoplasmic reticulum. JAK2 and TYK2 enhance the cell surface expression of the thrombopoietin receptors, and the expression of TYK2 is required for stable cell surface expression of IFN-αR1 and IL10R2.

Cytokine receptors compete for a limited amount of JAK as observed in IL-12/IFNα-1R system. A receptor without a kinase is unable to send signals and may dilute the effects of a cytokine by serving similar to an artificial receptor used clinically as decoys as is the case for IL-4 antagonist for the treatment of asthma.

3.9.2 Janus Kinase Inhibitors

Janus kinase inhibitors block the function of one or more Janus kinase family member and as a result inhibit JAK–STAT signaling pathway. Interference with this pathway will block cytokine signaling. These drugs have therapeutic application for the treatment of inflammatory diseases and cancer.

3.9.2.1 Tofacitinib (Xeljanz)

Tofacitinib, the first JAK inhibitor, is a specific inhibitor of JAK3 and interferes with the effector function of IL-2, IL-4, IL-15, and IL-21. Tofacitinib inhibits the differentiation of TH2 cells and is effective for the treatment of the allergic disease. To a lesser degree, it also inhibits JAK1 and JAK2 resulting in the inhibition of IL-6 and

IFN-ϒ signaling and therefore TH1 differentiation, though not yet approved by the FDA for this purpose. In the United States, tofacitinib is currently approved for the treatment of rheumatoid arthritis and is under investigation for the treatment of psoriasis, inflammatory bowel disease, rejection of tissue transplants, and other immunological diseases. For rheumatoid arthritis, it suppresses STAT1-dependent genes in the joint tissue. It can be used alone or in combination with methotrexate. For psoriasis, both oral and topically administered forms are being tested and both have shown improvement in treating the symptoms of the disease.

The drugs that may interact with tofacitinib include azathioprine, abatacept, anakinra, cyclosporine, etanercept, adalimumab, infliximab, tocilizumab, rituximab, and certolizumab. Any agent that interferes with cytochrome p450 system may affect its clearance.

The most common side effects reported are infections (throat, nose, sinus, urinary tract), headache, diarrhea, high cholesterol levels, throat irritation, and neutropenia. Other side effects such as fast heartbeat, unusual tiredness, severe stomach/abdominal pain, and signs of liver disease are also possible. A serious allergic reaction to drug is rare.

3.9.2.2 Ruxolitinib (Jakafi)

Ruxolitinib is a selective inhibitor of Janus kinases 1 and 2 (JAK1, JAK2). As described JAK1 and JAK2 are involved in the recruitment of signal transducers and activators of transcription (STATs) to cytokine receptors, resulting in the regulation of gene expression. Ruxolitinib is an inhibitor of impaired JAK signaling, which is associated with myelofibrosis. Myelofibrosis (osteomyelofibrosis) is a rare form of bone marrow cancer in which there is fibrosis or the replacement of the marrow with collagenous connective tissue fibers of an abnormal clone of hematopoietic progenitor cells. Ruxolitinib is used for the treatment of intermediate or high-risk myelofibrosis. It is also indicated for the treatment of polycythemia vera. Polycythemia vera is a disease where the bone marrow makes a high number of red cells, and this may also result in the overproduction of white blood cells and platelets. Furthermore, it is also under investigation for the treatment of pancreatic cancer, lymphoma, plaque psoriasis, and alopecia areata. Both oral and topical forms of ruxolitinib are also being tested to treat psoriasis.

Ruxolitinib is metabolized by CYP3A4 and to a lesser extent by CYP2C9, and the drugs interfering with this pathway interfere with its levels and clearance. For example, fluconazole and erythromycin increase its steady-state levels and rifampin will decrease its levels.

The side effects of ruxolitinib include herpes zoster, opportunistic infections, elevated cholesterol and abnormal alanine transaminase (ALT) and aspartate transaminase levels (AST), shortness of breath, pale skin, rapid heartbeat, trouble in concentrating, unusual bleeding (nose, mouth, vagina, rectum), easy bruising, purple or red pinpoint spots under the skin, flu-like symptoms, and vomiting. Fig. 3.5 depicts the mechanism of action of tofacitinib and ruxolitinib.

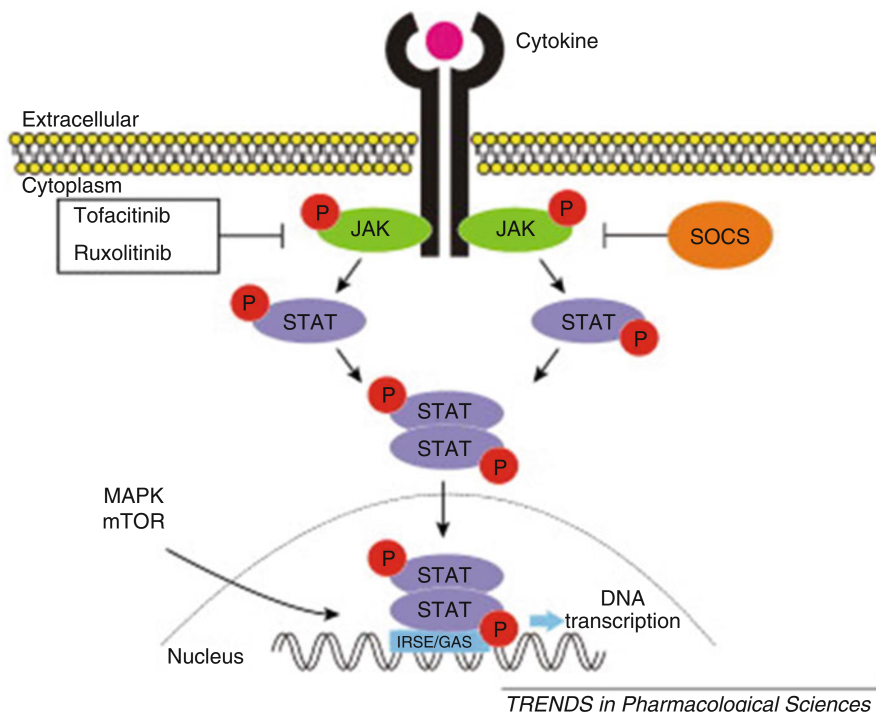

Fig. 3.5 Inhibition of JAK/STAT-dependent signaling pathways by tofacitinib and ruxolitinib (Source: Koenders MI, van den Berg WB (2015) Janus kinase inhibitors. *Trends in Pharmacological Sciences*. 36: 189–195. Elsevier, with permission)

3.10 Mitogen-Activated Protein Kinases

Mitogen-activated protein (MAP) kinases are signal transducers that play a pivotal role in a variety of cellular functions. Such kinases include a large family of protein kinases involved in multiple cellular functions including cell proliferation, differentiation, survival and death, and gene regulation. In addition to numerous biologic functions, they also play an important role in immune response and inflammation, and their aberration leads to disease state. Mitogen-activated protein kinases are part of signal transduction cascades that are involved in delivering extracellular message via cytoplasm to the nucleus. Following activation, these proteins migrate from the cytoplasm to the nucleus where they regulate gene expression by activating a number of proteins and transcription factors. For activation, they require phosphorylation on tyrosine and threonine in the conserved threonine–X–tyrosine sequence in kinase subdomain VIII. The tyrosine and serine-/threonine-specific phosphatases are involved in activation.

A MAP kinase module is composed of three kinases where MAP kinase kinase kinase (MAPKKK) will phosphorylate and induce a MAP kinase kinase (MAPKK),

which will then phosphorylate and activate a MAP kinase. MAP kinase either phosphorylates transcription factors that are non-kinase proteins or other kinases that are called MAP kinase-activating protein kinases (MKs). There are four distinct classes of MAP kinases, which include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases, P38 isoforms, and ERK5.

3.10.1 Extracellular Signal-Regulated Kinases

The ERK family is activated by mitogens and is critical in transducing signals, resulting in cell proliferation. ERK pathway consists of the MAPKKKs, A-Raf, B-Raf, C-Raf-1, the MAPKKs, MEK1, and MEK2; the MAPKs ERK1, and ERK2; and the MAPKAPKs, MNK1, MNK2, MSK1, MSK2, RSK1, RSK2, RSK3, P70S5K, and P70S6K. The sequence of the tripeptide motif for ERK is Thr-Glu-Tyr. Cytokines and growth factors activate A-Raf, B-Raf, and C-Raf-1, which then phosphorylate MEK1 and MEK2 resulting in their activation. ERK1 and ERK2 are then phosphorylated by MEK1 and MEK2. The targets of ERK1 and ERK2 include STATs, ELK-1, and Ets (Fig. 3.6).

3.10.2 c-Jun N-Terminal Kinases

The JNK/stress-activated protein kinases (SAPKs) do not respond well to mitogens but are strongly activated by agents that induce cellular stress. These kinases phosphorylate c-Jun transcription factor. The sequence of the tripeptide motif for JNK is Thr-Pro-Tyr. The activators of cytokine and tyrosine kinase receptors transduce signal to the upstream MAPKKKs. These MAPKKKs include ALK, MLK, TLP, and TAK, which phosphorylate MAPKKs, MKK4, and MKK7. The phosphorylated MKK4 and MKK7 then activate JNK1, JNK2, and JNK3 by phosphorylation (Fig. 3.7). These kinases play a significant role in apoptosis and immunologic diseases.

3.10.3 P38 Isoforms

As was the case for JNK/SAPKs, P38MAPKs do not respond well to mitogens but are strongly activated by agents that induce cellular stress. The P38 MAPKs include P38α, P38β, P38γ, and P38δ. All of the family members contain a sequence TGY in their activation loop. They share the upstream MPAKKK activators with c-Jun N-terminal kinases and are activated by cytokines, hormones, and environmental stress. The sequence of the tripeptide motif for P38 is Thr-Gly-Tyr. They phosphorylate MKK3 and MKK6. P38α and P38β are activated by MKK3; however, all

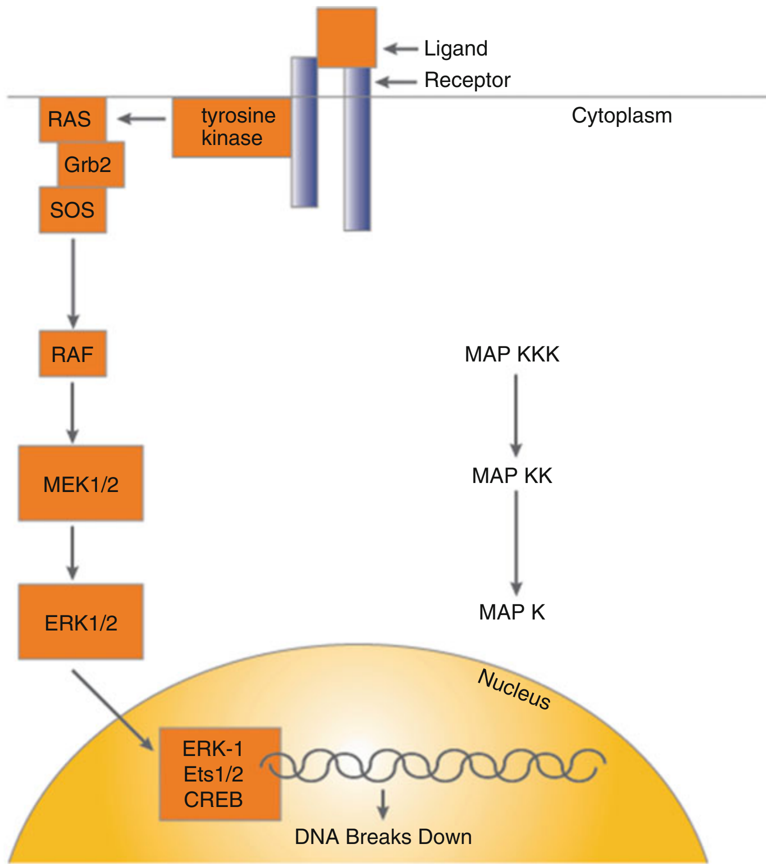

Fig. 3.6 The ERK/MAP kinase signaling pathway; cytokines and growth factors activate tyrosine kinase to which the adapter protein Grb2 binds. This localizes SOS to plasma membrane. Ras is then activated by SOS. Activated Ras then binds to RAF which forms a transient membrane-anchoring signal. Active RAF kinase phosphorylates MEK. The activated MEK phosphorylates ERK1/ERK2 which also migrates to the nucleus to phosphorylate ELK-1, Ets1/2, and CREB, resulting in the activation and expression of respective genes

P38 MAP kinases are activated by MKK6 (Fig. 3.8). Among other biological functions, they play an important role in the immune response.

3.10.4 Extracellular Signal-Regulated Kinases 5

ERK5 is similar to ERK1/ERK2 and has the Thr-Glu-Tyr (TEY) activation motif. It is activated by growth factors, mitogens, and oxidative stress. MEK5, upstream activator of ERK5, is activated by MEKK2 and MEKK3. MEK5 phosphorylates and

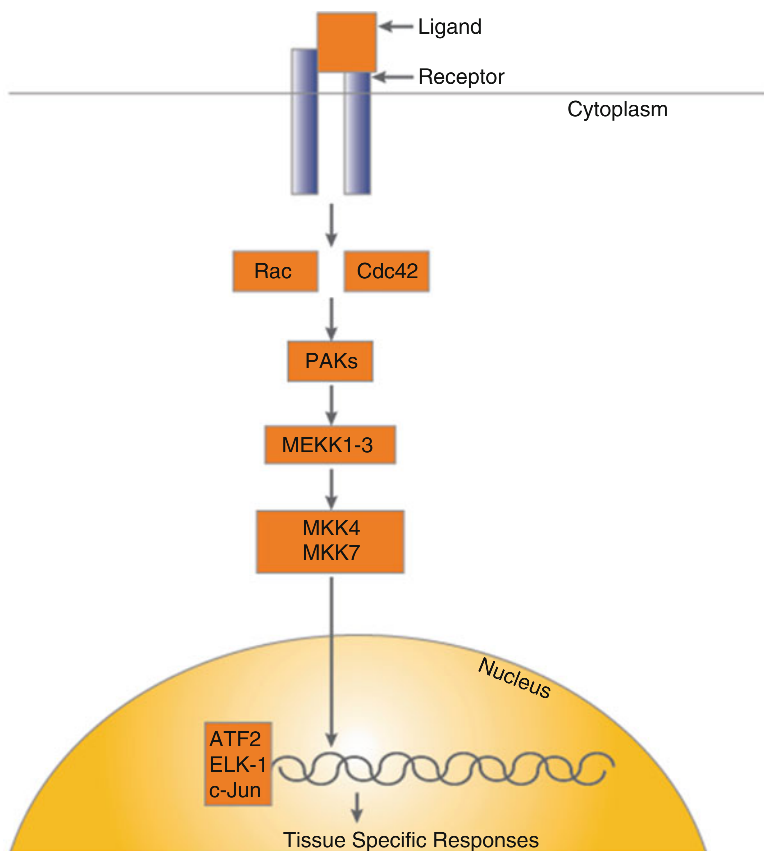

Fig. 3.7 The JNK/SAPK signaling pathway; a variety of signals including cytokines activate the mitogen-activated protein kinases (MAP kinases). The JNK/SAPK cascade is activated in response to inflammatory cytokines, heat shock, or ultraviolet radiation. Two small G-proteins, Rac and cdc42, mediate the activation of the MAP kinases. After activation cdc42 binds to and activates PAK65 protein kinase. This results in the activation of MEKK which eventually phosphorylates JNK/SAPK that migrates to the nucleus and activates the expression of several genes specifically the phosphorylation of c-Jun

induces ERK5. The pathway involving MAPK ERK5 mediates growth factor and stress-induced signal transduction. It is a contributor to cell survival mechanisms. The inhibitors of ERK1/2 (classical MAP kinases) also inhibit ERK5. It mediates the effects of several oncogenes and its abnormal levels are associated with some forms of cancer. Selective activation of ERK5 induces a reporter gene driven by the IL-2 promoter without affecting CD69 expression.

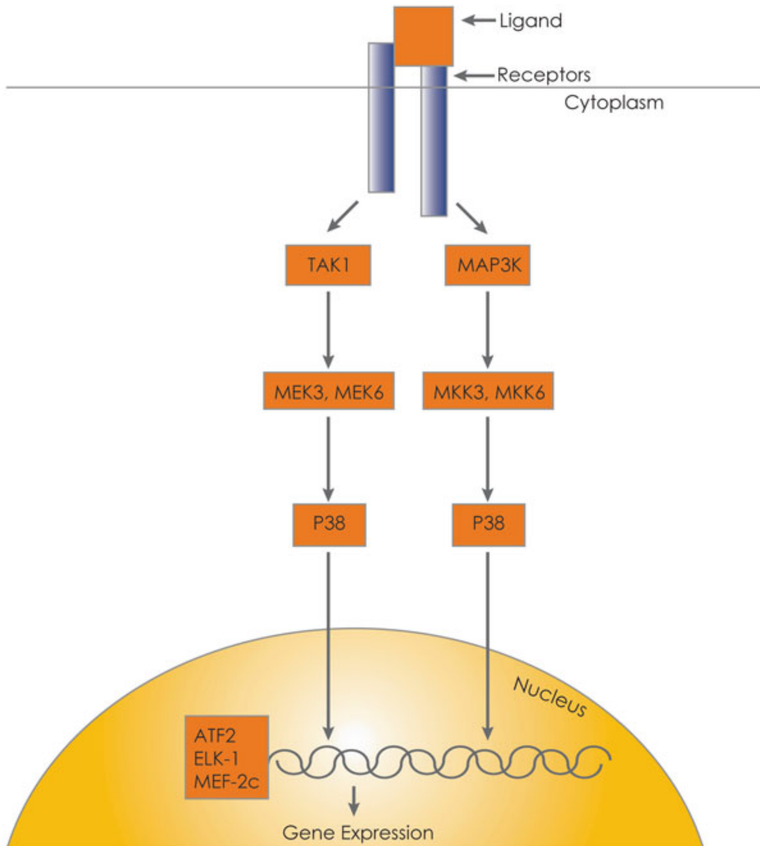

Fig. 3.8 The p38 kinase signaling pathway; inflammatory cytokines, osmotic stress, and endotoxins activate this signaling pathway. The maximum activation of p38 requires two MAP2Ks, MKK3 and MKK6. Following activation p38 translocates to the nucleus and phosphorylates ATF2, ELK1, and MEF-2C. P38 can also be activated independent of MAP2Ks by TAK1 binding protein via TAB1

3.11 Mitogen-Activated Protein Kinases in Immune Response

Toll-like receptors through intermediate signals including MyD88 adapter protein, IRAK, TRAF6, and others cause the activation of MAP kinases and NF-κB resulting in the production of inflammatory cytokines, which include IL-1, IL-12, and TNF-α. The P38 MAPK pathway plays an important role in the production of IL-12 in dendritic cells and macrophages. In dendritic cells, a receptor–ligand pair upstream of P38 and JNK, CD40-CD40L interaction, is responsible for inducing

IL-12 production. P38 MAPK also regulates TNF-α biosynthesis via MAP kinase-activated protein kinases (MPAKAP), MK2, and MK3, at the posttranscriptional level. AU-rich elements (ARE) in TNF-α transcripts (3′-untranslated) are involved in the regulation of the translation of TNF-α by P38.

TNF-α and IL-1 activate both JNK and P38 MAP kinases; P38α plays a significant role in IL-1-mediated inflammatory responses. However, TNF, but not IL-1, requires MKK3-P38 for its cellular effects. The production of IL-6 and type-1 interferons requires JNK, which is also required for IL-1-induced effects in rheumatoid arthritis including joint inflammation and collagenase-3 expression. In IL-1 signaling pathway, JNK is activated by TRAF6- and TRAF2- induced JNK after activation of TNF receptors. While both JNK kinases, MKK4 and MKK7, are involved in stress-induced JNK activation, only MKK4 is involved in JNK activation following MEF treatment with IL-1 or TNF-α. The phosphorylation site in the sequence of the tripeptide motif is different for MKK4 and MKK7, as MKK7 phosphorylates threonine, whereas MKK4 preferentially phosphorylates tyrosine.

3.12 Effects of Mitogen-Activated Protein Kinases on T Cells

Induction of ERK is important for T-cell activation. The interaction of antigen with TCR in context with MHC molecules results in the recruitment of various molecules including Grab2 and SLP76, both adopter proteins to the cell surface. This results in the induction of SOS-Ras-MEK-ERK pathway. ERK pathway has also been implicated in thymocyte selection and maturation, TH2 differentiation, and IL-4-induced STAT6 and IL-4 receptor phosphorylation.

P38 MAP kinases play a role in TH1 differentiation as IL-12 and IL-18 are involved in P38 activation in T lymphocytes. INF-γ produced by TH1 cells is inhibited by imidazole antagonists of P38 kinases, which do not affect IL-4 secretion by TH2 cells, but INF expression is dependent on P38 regulation.

Naïve T cells have very limited JNK activity; however, its activity and expression are significantly augmented when T cells are activated, reaching a peak after 30–60 min of activation. Activation of JNK is also dependent on signals from activated TCR. While not essential for the induction of JNK activity, CD28 costimulation significantly enhances its activity. JNK also may have an important role in TH1 function where it is highly activated as opposed to TH2 cells. JNK2 is essential for cytokine production and TH cell differentiation. In the absence of JNK2, JNK activity in TH1 cells is reduced. Both JNK and P38 play an important role in the differentiation of TH1 cells. This causes a feedback amplification of signal strength for JNK and P38 pathways resulting in the induction of Rac2 and GADD45. Rac2 is an upstream effector for JNK and P38 pathways, whereas GADD45 is involved in JNK signaling, which is specific to TH1 cells. The induction of Rac2 and GADD45 significantly augments the signaling of JNK and P38, resulting in effector cytokine production by TH1 cells.

3.13 Clinical Potential of Targeting MAPK

ERK signaling pathway plays a crucial role in cell proliferation and therefore its inhibitors may have therapeutic potential as antineoplastic drugs. The signaling by ERK1/2 is associated with several proto-oncogenic "driver" mutations. These include constitutively active/mutant receptor tyrosine kinases, Raf, or Ras proteins. In obese individuals resistance to insulin may involve a role of c-Jun N-terminal kinases. A few JNK inhibitors are under clinical development for diverse diseases.

3.13.1 Sorafenib (Nexavar)

Sorafenib is a tyrosine kinase inhibitor (TKI) associated with vascular endothelial growth factor receptors (VEFRF), platelet-derived growth factor receptors (PDGFR), and Raf (rapidly accelerated fibrosarcoma) kinases. Raf kinases are composed of three serine/threonine-specific protein kinases that are related to retroviral oncogenes. The three RAF kinase family members include A-Raf, B-Raf, and C-Raf. Sorafenib more avidly affects C-Raf than B-Raf. Raf kinases play a role in the Ras-RAF-MEK-ERK-signal transduction cascade, also called as mitogen-activated protein kinase (MAPK) cascade. Furthermore, sorafenib has an inhibitory effect on intracellular serine/threonine kinases.

It is indicated for the treatment of advanced renal cell carcinoma, hepatocellular carcinoma, and thyroid cancer. Sorafenib is in clinical trials to treat desmoid tumors (aggressive fibromatosis). The treatment with sorafenib causes autophagy, resulting in inhibition of tumor growth. Autophagy is a catabolic mechanism, which causes the degradation of unnecessary cellular parts or organelles via the effects of lysosomes. During the disease, autophagy is responsible for both the cell survival and the promotion of cell death. Autophagy can also promote drug resistance. When compared with placebo, sorafenib prolonged progression-free survival in advanced clear cell renal carcinoma patients, who were refractory to prior treatments. In patients treated for advanced hepatocellular carcinoma with sorafenib, the median survival and the time for radiological progression are about 3 months longer when compared with placebo. Sorafenib has also been examined as adjuvant therapy in high-risk liver transplant recipients undergoing the procedure for hepatocellular carcinoma. The results in a rather limited sample have shown that this is a safe, feasible, and well-tolerated intervention.

The side effects of sorafenib include skin rash, diarrhea, fatigue, weakness, itching, hair loss, dry mouth, weight loss, headache, numbness in hands and feet, and dry or peeling skin. The more adverse and serious side effects include pancreatic atrophy, skin hemorrhage, leukopenia, neutropenia, anemia, thrombocytopenia, sei-

zures, bloody vomiting, confusion, congestive heart failure, myocardial infarction, and renal failure. The side effects need to be carefully monitored, if the patient is taking neomycin, phenobarbital, rifampin, anticoagulants, carbamazepine, dexamethasone, dofetilide, dronedarone, ibutilide, irinotecan, phenytoin, procainamide, quinidine, warfarin, and/or amiodarone.

3.14 Cell Surface Signals Activate Extracellular Signal-Regulated Kinases 1/2 and Other Mitogen-Activated Protein Kinases

Tyrosine kinase pathway is one of the most well-defined cell signaling systems that transduces messages from cell surface receptors to ERK1/2 transcription factors. Binding of the agonist to the cell surface receptors causes the autophosphorylation of the tyrosine residues in the receptor. Phosphorylation of the receptors initiates downstream signaling pathways. This results in the activation of a G-protein Ras, which involves the recruitment of adopter proteins shc and Gγb2 to the receptor and formation of a complex following linkage among SH2 domains and phosphotyrosine residues. This complex then incorporates the guanine nucleotide exchange factor (GEF), Son of Sevenless, resulting in the induction of Ras. The ultimate result is the exchange of GDP for GTP by Ras as GTP-liganded Ras can interact with Raf isoforms. This results in Raf-1-mediated signaling. G-protein (Gαs)-mediated signaling has multiple effects on ERK activity, some of which are cAMP dependent. PKA that is cAMP dependent, inhibits Raf-1 activity.

3.15 Nuclear Factor-κB

NF-κB is a family of pleiotropic transcription factors that regulate the expression of a wide variety of genes. These genes are involved in a variety of biological functions including the immune and inflammatory response. The classical activated form of NF-κB is a P50/P65 heterodimer. Generally, NF-κB is present in an inactive form in the cytoplasm where it is noncovalently associated with its inhibitor, IκB. This inhibitor prevents NF-κB-mediated signal transduction, which involves its migration to the nucleus and induction of specific gene expression after binding to the DNA. Some cytokines including IL-1 and TNF-α after binding to their specific cell surface receptors cause the phosphorylation of IκB, resulting in its degradation and removing the inactivity barrier from NF-κB. This permits

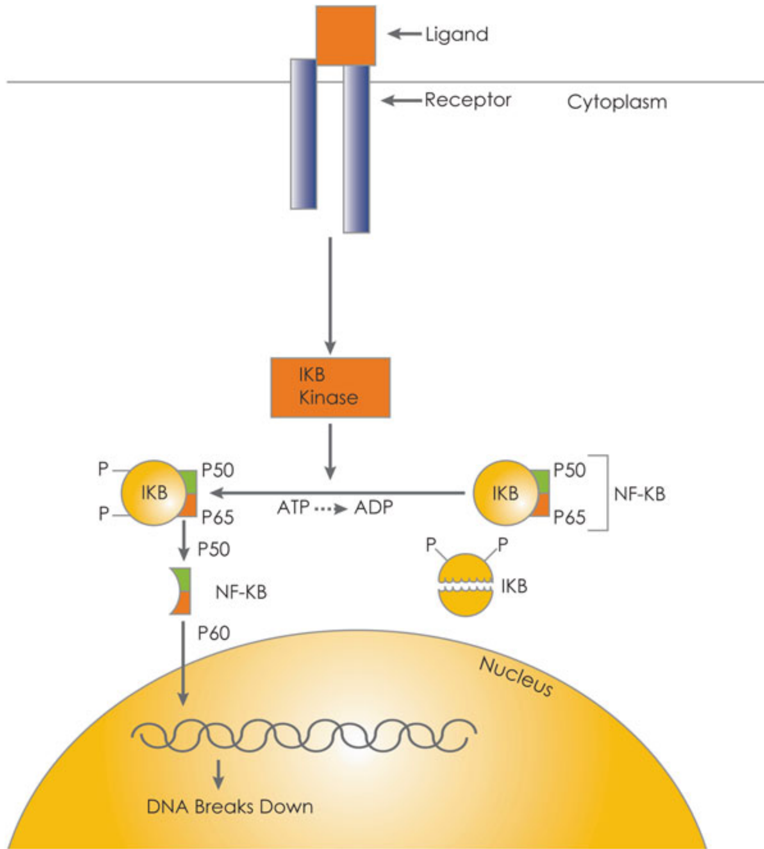

Fig. 3.9 The NF-κB signaling pathway: NF-κB is a P50/P65 heterodimer which is present in the cytoplasm in an inactive form as it is non-covalently associated with IκB. IκB is an inhibitor of NF-κB and prevents its migration and gene expression signal in the nucleus. Selected cytokines after binding to their specific receptors phosphorylate IκB resulting in its degradation which removes the inactivity barrier from NF-κB. This is followed by the migration of NF-κB into the nucleus where it binds to specific genes and results in their induction and gene expression

the migration of NF-κB into the nucleus where it binds to specific genes in the DNA resulting in their induction and expression (Fig. 3.9). One gene regulated by NF-κB is IκBα, which serves as a feedback inhibitor to maintain homeostasis. IL-1 and TNF-α induce NF-κB in a biphasic manner that includes a transient phase and a persistent phase. The transient phase is mediated via IκBα and the persistent phase is mediated via IκBβ. The activation of NF-κB is complex and involves the activation of IκB kinase (IKK), which phosphorylates IκB that is bound to NF-κB. Other transcription factors involved in this signaling cascade are cell dependent.

3.16 Inhibitors of Cytokine Cell Signaling

Cytokine signaling is strictly regulated at several sites to avoid damages resulting from uncontrolled signal transduction initiated from activated cytokine receptors. A number of cytokine signaling inhibitors have been described.

3.16.1 SH2-Containing Phosphatase Proteins (SHP)

SH2-containing phosphatase proteins are expressed constitutively and are inhibitors of cytokine-mediated signal transduction. They act by dephosphorylating multiple transcription factors including JAKs and their receptors. Mammalian SHP have two family members, SHP1 and SHP2, which are composed of two repetitive N-terminal SH2 domains and a C-terminal protein tyrosine phosphatase domain. In general, activation of intermediate transcription factors involves the phosphorylation of serine, tyrosine, or threonine residues. The phosphotyrosine residues of a variety of cytokine receptors are targets of SHP1 and SHP2 via their SH2 domain. The signaling components that are inhibited by SHP1 after dephosphorylation include transcription factors for IL-4 receptors, JAK-2, erythropoietin receptors, and stem cell factor receptor (c-Kit). The role of SHP2 is that of an inducer of transduction pathway. However, it has been suggested that SHP2 can downregulate cytokine receptor-mediated signal transduction mechanisms through gp130 receptors.

3.16.2 Protein Inhibitors of Activated Signal Transducers and Activators of Transcription (PIAS)

Protein inhibitors of activated STATs are constitutively expressed, and their mechanism of action is inhibition of STAT pathways by sumoylation. The PIAS family members, PIAS1 and PIASx, inhibit STAT1, and PIAS3 and PIASy inhibit STAT3 and STAT4, respectively. There is a high degree of sequence conservation among these four family members. Other family members also exist, hZIMP7 and hZIMP10, but have limited similarity with other members. PIAS are transcriptional coregulators that mediate their effects by at least two mechanisms; they act as SUMOE3 ligases and increase sumoylation of the substrate resulting in the addition of SUMO moieties and consequently modifying their properties, while the other mechanism of action is independent of sumoylation. They act either by suppression or induction, which depends on the target transcriptional factor. One of the resultant effects is the relocalization of transcriptional regulators to different subnuclear compartments.

3.17 Suppressors of Cytokine Signaling (SOCS)

Suppressors of cytokine signaling include eight members (SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7, and CIS), which are composed of a central SH2 (Src homology 2) domain, an N-terminal domain, and a C-terminal SOC box domain. The N-terminal domain has variable length whereas the C-terminal domain is composed of 40 amino acid sequence. The SH2 domain, in phosphotyrosine residues of cytokine receptors, is the target of SOCS proteins. These family members bind to domains of cytokine receptors that include SOCS2, SOCS3, and CIS. Alternatively, SOC1 binds to its target sites of JAKs. The cytokine signaling is suppressed by multiple mechanisms, which include competition with STATs for the sites of receptor phosphorylation, inhibition of JAKs activity, and/or proteasomal degradation after binding to signaling proteins. SOCS proteins are depicted as key negative regulators of cytokine signaling, most important of which is the inhibition of JAK/STAT pathway. Their synthesis is induced by a variety of signals including IL-6, TNF, TGF-β, and LPS.

SOCS2 and SOCS3 are also the negative regulators of cytokine receptor-mediated signal transduction. All three SOCS, SOCS3 in particular, inhibit the signals of hematopoietin class of cytokine receptors. SOCS3 inhibits a variety of cytokine signals including IL-2Rβ, gp130 receptors, GHR, and EPOR and also regulates intestinal inflammation and energy homeostasis by leptin. It is also involved in the regulation of EPO signaling. Cytokine receptors aggregate after binding to their respective agonist, resulting in the activation of Janus kinases. Induction of Janus kinases causes receptor tyrosine phosphorylation and also of other proteins involved in signaling. This sequence also results in the activation of other signaling molecules such as the signal transducers and activators of transcription (STATs). In contrast to SOCS1, which directly binds to the JAK kinases and inhibits their catalytic activity, CIS binds to the cytokine receptor, resulting in the inhibition of STATs activity. SOCS3 acts by both mechanisms described for SOCS1 and CIS. SOCS3 does not possess a high affinity for JAKs and requires cytokine receptors to inhibit JAK kinase activity. The interaction of SOCS3 with the activated cytokine receptor is required, before recruitment to the signaling complex takes place, which results in the inhibition of cytokine-mediated activity, after it binds to JAK kinases. Other molecules are also degraded by SOCS proteins including the interaction of SOCS box domain with Elongins B and C, and these proteins via proteasomes target proteins for destruction, suggesting that SOCS proteins regulate signaling proteins and inhibit their catalytic activity as well as recruitment.

The transcriptional upregulation of SOCS1, SOCS3, and CIS is caused by STATs as they regulate the expression of SOCS1, and the binding sites for STAT1, STAT3, and STAT6 are present in the SOCS1 promoter. IL-6 or LIF-induced expression of the SOCS1 RNA is inhibited by a dominant negative version of STAT3. Fibroblasts lacking STAT1 are unable to induce expression of the SOCS1 mRNA in response to

IFN-γ. The effect of STAT1 is indirect on the SOCS1 gene as it is mediated via interferon regulator factor-1 (IRF-1). Signal transducers and activators of transcription also regulate the expression of the SOCS3 gene. STAT1/STAT3 binding element is present in the SOCS3 promoter that is responsible for LIF-induced activation of the SOCS3 promoter. Furthermore, SOCS3 expression is regulated by STAT5B. Four STAT5 binding sites are present in the promoter of CIS gene, which is completely responsible for its activation by EPO.

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Chapter 4
Immunosuppressive Agents

Abstract Immunosuppression is challenging, since the immunosuppressive drugs do not produce the same effects on all immune responses. The doses required to inhibit the immune response may also differ from antigen to antigen. Furthermore, the primary immune responses can be more easily and more effectively suppressed than the secondary immune responses. These drugs need to be used on a lifelong basis and cause major adverse reactions. In this chapter, the focus will be on the specific immunosuppressive agents. They include calcineurin inhibitors, mTOR inhibitors, sphingosine-1-phosphate receptor (S1P-R) modulator, and inhibitors of the costimulatory molecules for antigen recognition. In addition, specific and non-specific immunosuppressive cytotoxic agents will be described. Lastly glucocorticoids, antithymocyte and antilymphocyte globulins, Rho (D) immune globulin, and polyclonal antibodies will be discussed. The major focus will be on the pharmacology of these drugs, which will include their mechanism of action, pharmacokinetics, drug–drug interactions, toxicity, and clinical uses.

Keywords Immunosuppression • Calcineurin inhibitors • Cyclosporine • NFATc • NFATn • Cyclophilins • Tacrolimus • mTOR inhibitors • Sirolimus • PP2-A • IL-2 receptors • Cell cycle • FK506-binding protein-12-rapamycin-associated protein 1(FRAP1) • Serine threonine kinase • Phosphatidylinositol-3-kinase (PI3K) • PI3K/AKT pathway • mTORC1 • mTOR/PI3K/AKT pathway • Nephrotoxicity • Everolimus • Promus everolimus-eluting coronary stent system • Chronic allograft vasculopathy • Sphingosine-1-phosphate receptor (S1P-R) modulator • Fingolimod • Sphingosine-1-phosphate (SIP) • Adherens • P21-activated kinase-1 (Pak1) • Cdc42 • Rac1 • Belatacept • CTLA-4 • CD152 • CD28 • CD80/86 • Tissue transplantation • Fused Fc region of the antibody IgG1 • Abatacept • Mycophenolate mofetil • Purine biosynthesis • De novo pathway • Salvage pathway • Mycophenolic acid • Azathioprine • Cyclophosphamide • Methotrexate • Glucocorticoids • Antithymocyte and antilymphocyte globulins • Rho (D) immune globulin • Muromonoab-CD3 • Daclizumab • Basiliximab

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4.1 Introduction

Successful organ transplantation requires effective immunosuppression. Most of the modern advances in tissue transplantation have resulted from our precise understanding of the immune mechanisms involved in tissue rejection as well as from advances in surgical techniques to some degree. The last two decades have witnessed the introduction of several potent immunosuppressive agents, which have led to considerable success in transplant medicine. The development of effective immunosuppressive therapy has been essential in tissue transplantation and for improvements in its positive outcome. Great advances have been made over the past 50 years in organ transplantation despite the complexities involved in immune suppression after tissue transplantation. The development of novel immunosuppressive agents has increased the graft survival rates; however, the side effects of these drugs resulting from their long-term use continue to present significant challenges. In addition to organ transplantation, immunosuppressive agents have found their use in the prevention of Rh hemolytic disease of the newborn and for the treatment of some autoimmune diseases.

The initial organ transplantation was performed in 1933 when a kidney was transplanted from a cadaver. Total lymphoid irradiation was used for the immune suppression, but the tissue was rejected and the patient eventually died. This was followed by the use of corticosteroids as immunosuppressive agents, but unfortunately steroids by themselves also did not produce positive results. In the early 1960s, cytotoxic agents were introduced for immune suppression; these were followed by the use of a combination of cytotoxic agents and corticosteroids until the mid-1980s when cyclosporine was discovered by Borel.

Despite all the advances in surgical techniques and the development of newer immunosuppressive drugs and antibodies, long-term survival of patients with organ transplants continues to provide a challenge for the clinicians. The complexity of managing immunosuppression arises from many diverse factors related to the development of immune responses, for example, the differential effects of immunosuppressive agents on primary versus secondary immune responses (they are more effective against the primary immune response). The primary immune response is observed during antigen processing, antigen-induced initial T-cell proliferation, and cytokine production. The secondary immune response is based on immunologic memory, which results in robust tissue rejection, and less impressive effects of immunosuppressive agents are observed. Another challenge that the immunosuppressive agents face is that their effects are antigen dependent, that is, they could vary from antigen to antigen and require constant adjustments of the doses of the same drug to manage immune suppression.

The major classes of immunosuppressive drugs employed in clinical practice to avoid tissue rejection include calcineurin inhibitors, TOR inhibitors, cytotoxic agents, glucocorticoids, and monoclonal antibodies. These drugs need to be used on a lifelong basis and have major undesirable side effects.

4.2 Calcineurin Inhibitors

4.2.1 Cyclosporine

Specific immunosuppressive therapy has its origin in the 1970s, when cyclosporine was discovered by J. F. Borel in 1976. The drug was originally considered as an antifungal compound but its immunoregulatory activities were learned rapidly. The discovery was a result of a program of a Swiss pharmaceutical company to discover new antibiotics from fungal metabolites. Cyclosporine was isolated from fungus *Tolypocladium inflatum Gams*. Its initial isolation was from a Norwegian soil sample, and its chemical analysis revealed that cyclosporine was a cyclic undecapeptide. The structure and conformational analysis was done with chemical degradation, X-ray crystallography, and nuclear magnetic resonance imaging. Cyclosporine is a hydrophobic compound insoluble in water but soluble in other organic solvents.

4.2.1.1 Mechanism of Action

Cyclosporine enters through the cell membrane and binds to cyclophilins in the cytoplasm. Cyclophilins are a family of small proteins that selectively bind cyclosporine as well as its active analogs. Their distribution is abundant in lymphoid cells but they are present in most of the human tissue. The cyclosporine binding to cyclophilin results in the formation of a cyclosporine–cyclophilin complex. During an immune response, activation of T-cell receptor results in an increase in intracellular Ca^{2+} that activates calcineurin, an enzyme called serine/threonine phosphatase, which is calcium dependent. Under physiologic conditions, calcineurin after its activation by Ca^{2+} dephosphorylates a cytosolic component of the nuclear factor of activated T cells (NFATc). After its dephosphorylation, NFATc migrates from the cytoplasm to the nucleus where it associates with the nuclear component of the nuclear factor of activated T cells (NFATn). This association of the NFATc and NFATn results in the activation of the transcription of a number of genes including cytokine genes for IL-2, IL-3, IL-4, TNFα, GMCSF, and others.

After the administration of cyclosporine, cyclosporine-cyclophilin complex is formed, which binds to calcineurin, resulting in its inability to dephosphorylate NFATc, and as a result, the transport of NFATc to the nucleus is prevented, and consequently its association with NFATn does not proceed (Fig. 4.1). The association of NFATc with NFATn is essential for the initiation of IL-2 production that is achieved through binding of NFATc–NFATn to the promoter of interleukin-2 (IL-2) gene. As a result, IL-2 production is inhibited, which is necessary for the optimal function of the immune response. Cyclosporine does not inhibit cytokine-induced transduction mechanisms and also has no effect on antigen recognition by T cells in context with MHC molecules.

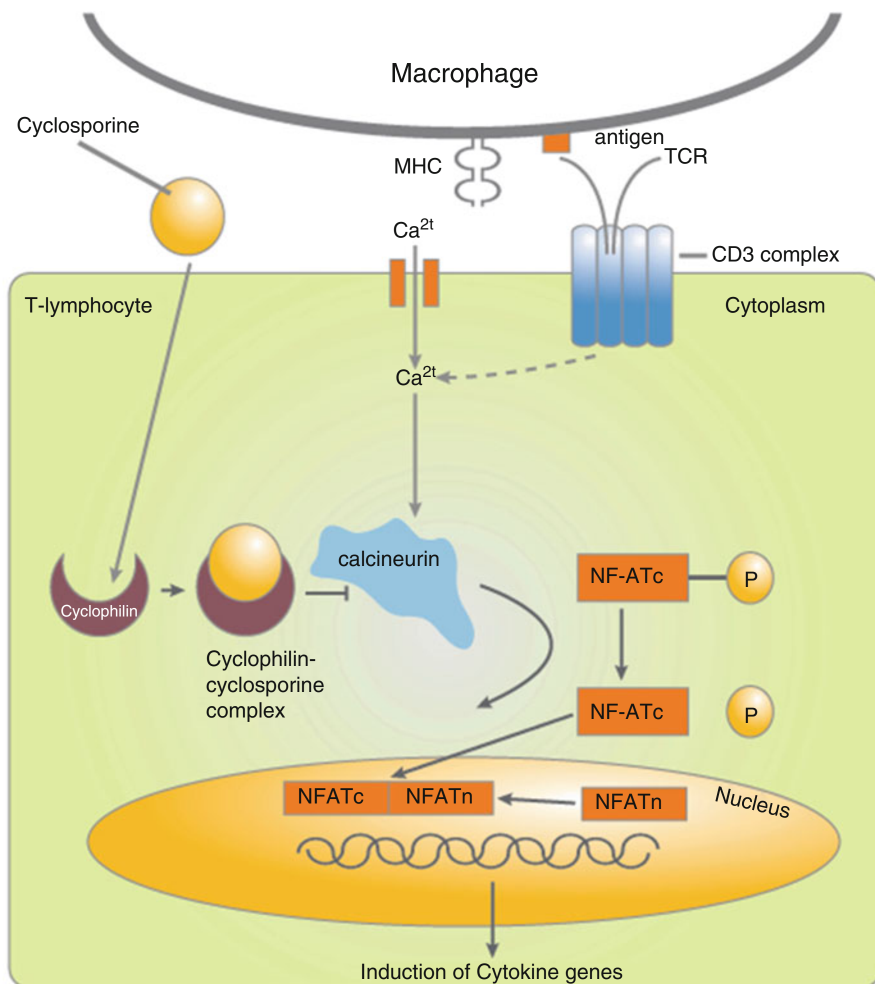

Fig. 4.1 Mechanism of action of cyclosporine: cyclosporine readily diffuses into the cytoplasm of the target cells where it binds to cyclophilins. The cyclosporine-cyclophilin complex stably associated with calcineurin and inhibits calcineurin activity. Calcineurin is a Ca2+- dependent enzyme, serine/threonine phosphatase which after activation by Ca2+ dephosphorylates a cytosolic component of NFAT (NFATc, cytosolic factor of activated T cells). After dephosphorylation NFATc migrate from the cytoplasm to the nucleus where it associates with NFATn (nuclear factor of activated T cells) and induces transcription of several cytokine genes including IL-2. Cyclosporin inhibits calcineurin activity after associating with cyclophilins resulting in the inhibition of IL-2 production and other cytokines

4.2.1.2 Absorption, Distribution, and Excretion

Cyclosporine is given orally or intravenously. Its oral bioavailability varies from 20 % to 50 %, and the peak concentrations in plasma are achieved within 3–4 h after its administration. Erythrocytes bind to about 70 % of the drug. It is significant to note that about 15–20 % cyclosporine is contained in leukocytes despite their small

content in total blood. Cyclosporine is extensively distributed in compartments other than the vasculature that may result in some of its toxic side effects. The half-life of cyclosporine is approximately 6 h. It is metabolized predominantly in the liver by CYP3A and is excreted in the bile. Only negligible amounts of the drug and its metabolites appear in the urine. More than 20 metabolites of cyclosporine have been identified, but the metabolites have far less pharmacologic activity and toxicity as opposed to the parent drug. Inhaled cyclosporine has been used after lung transplantation that has helped avoid its undesirable side effects and may at least delay the onset of obliterative bronchiolitis.

4.2.1.3 Drug Interactions

The blood concentrations of cyclosporine are impacted by any drug that acts on microsomal enzymes, particularly the CYP3A system. The drugs, which inhibit this enzyme would reduce the metabolism of cyclosporine and consequently will increase its blood concentrations. These drugs include antifungal agents, antibiotics, glucocorticoids, calcium channel blockers, protease inhibitors, and others. In contrast, the drugs that augment CYP3A activity will increase the metabolism of cyclosporine, resulting in reduced blood concentrations. These drugs include phenytoin, phenobarbital, trimethoprim–sulfamethoxazole, and rifampin.

4.2.1.4 Toxicity

The major side effects of cyclosporine are renal and nephrotoxicity, which are seen in about 25–75 % of patients. This could reduce glomerular filtration rate and renal plasma flow. There is also damage to proximal tubules and endothelial cells of small blood vessels. It causes hyperuricemia, which could result in hypercholesterolemia, increase in P-glycoprotein activity, and worsening of gout. Hypertension is seen in most of the cardiac transplant patients and in about 50 % of the renal transplants. About half of the patients receiving cyclosporine have elevated hepatic transaminase activity or concentration of bilirubin in plasma. Hirsutism and gingival hypoplasia are observed in 10–30 % of the patients taking cyclosporine. Other side effects include peptic ulcers, pancreatitis, gum hyperplasia, convulsions, breathing difficulties, fever, vomiting, and confusion. There is an increased vulnerability to fungal and viral infections, but there is a low incidence of malignancies if cyclosporine is administered alone and not with other immunosuppressive agents.

4.2.1.5 Clinical Uses

Cyclosporine is used to prevent organ rejection after tissue transplantation. It is also used for rheumatoid arthritis, psoriasis, and dry eyes (keratoconjunctivitis sicca). For tissue transplantation, it is mostly used in combination with other

immunosuppressive agents, and its doses vary based on the clinical circumstances. Its nephrotoxicity limits its use before the tissue is grafted, which poses a challenge in renal transplants where rejection must be differentiated from renal toxicity.

4.2.2 Tacrolimus

Tacrolimus is a 23-membered lactone chain isolated in 1984 from *Streptomyces tsukubaensis*, although it was originally found in a soil fungus. It is a macrolide antibiotic, and its name is derived from "Tsukuba macrolide immunosuppressant."

4.2.2.1 Mechanism of Action

Tacrolimus suppresses peptidyl-prolyl isomerase activity by binding to the immunophilin FK506-binding protein-12 (FKBP-12), and the tacrolimus-FKBP-12 complex binds to calcineurin and inhibits calcineurin phosphatase activity. As a result, calcineurin is unable to dephosphorylate NFATc, and thus, its migration to nucleus is blocked where its association with NFATn is necessary for the activation of key cytokine genes. Therefore, its mechanism of action is similar to cyclosporine although tacrolimus binds to a separate set of immunophilins in the cytoplasm. Tacrolimus, like cyclosporine, inhibits the secretion of key cytokines and inhibits T-cell activation (Fig. 4.2).

4.2.2.2 Absorption, Distribution, and Excretion

Tacrolimus is given orally (twice-daily dose regimen) or as an injection. A modified release (MR) oral dosage form of tacrolimus has been developed for administration once a day to overcome noncompliance, which is the major problem in acute graft rejection in solid transplant recipients. Tacrolimus is not completely absorbed by the GI tract and its rate of absorption could vary. It binds to plasma protein at a rate of 75–99 % with a half-life of approximately 12 h and is predominantly metabolized in the liver by CYP3A. Some of its metabolites have immunosuppressive activity. Most of tacrolimus is excreted in feces, and a negligible amount (<1 %) is excreted in urine without undergoing any metabolism.

4.2.2.3 Drug Interactions

As for cyclosporine, its blood concentration is impacted by drugs that act on the CYP3A system. The drugs, which inhibit this enzyme will increase its blood concentration, and the drugs that enhance the activity of CYP3A will decrease the blood concentration of tacrolimus. In combination with cyclosporine, it produces additive renal toxicity.

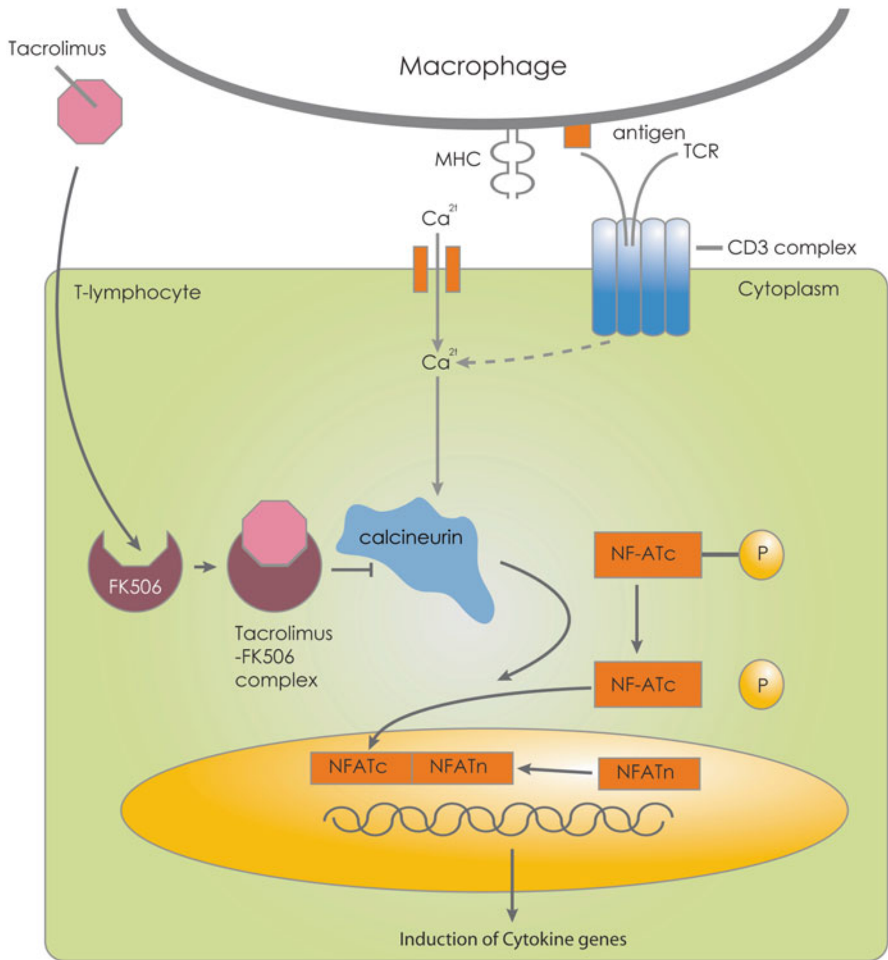

Fig. 4.2 The mechanism of action of tacrolimus: tacrolimus readily diffuses into the cytoplasm of the target cell where it binds to immunophilins (FK506-BP). The tacrolimus-immunophilin complex stably associated with calcineurin and inhibits calcineurin activity. Calcineurin is a Ca2+-dependent enzyme, serine/threonine phosphatase which after activation by Ca2+ dephosphorylates a cytosolic component of NFAT (NFATc, cytosolic factor of activated T cells). After dephosphorylation NFATc migrate from the cytoplasm to the nucleus where it associates with NFATn (nuclear factor of activated T cells) and induces transcription of several cytokine genes including IL-2. Tacrolimus inhibits calcineurin activity after associating with immunophilins resulting in the inhibition of IL-2 production and other cytokines

4.2.2.4 Toxicity

The side effects associated with tacrolimus administration include nephro- and hepatotoxicity, hypertension, tremors, seizures, diabetes mellitus, neuropathy, blurred vision, depression, loss of appetite, and confusion. Tacrolimus may cause opportunistic infection and could increase the severity of pre-existing infections, including

fungal or viral (herpes) infections. It has no effect on LDL cholesterol or uric acid. As was the case with cyclosporine, the administration of tacrolimus in combination with other immunosuppressive agents may increase the risk of tumors.

4.2.2.5 Clinical Uses

The immunosuppressive properties of tacrolimus are similar to cyclosporine, but it is more potent than cyclosporine, so lower doses are required. Its primary use is for preventing rejection after allogeneic transplants to reduce the risk of organ rejection. Tacrolimus is also administered to patients who are showing signs of rejection despite treatment with cyclosporine. Tacrolimus is also used for the treatment of severe atopic dermatitis and for severe refractory uveitis.

4.3 TOR Inhibitors

4.3.1 Sirolimus (Rapamycin)

Sirolimus is a macrolide antibiotic (macrocyclic lactone) first isolated from the soil samples of Easter Island as a product of the bacterium *Streptomyce*s *hygroscopicus* and shares a lot of its structure with tacrolimus.

4.3.1.1 Mechanism of Action

Sirolimus binds to the cytosolic protein FK-binding protein R (FKBP-12) but does not block calcineurin activity. It does not bind to cyclophilins, which are cytosolic receptors for cyclosporine. Unlike cyclosporine and tacrolimus, sirolimus does not inhibit the activation of NFAT responsive genes. After binding to its cytosolic receptors, sirolimus inhibits a protein kinase, the mammalian target of rapamycin (mTOR) pathway, via suppression of protein phosphatase 2 (PP2-A). When mTOR is inhibited, the cells will not proceed to the S phase, and cell cycle will be blocked (Fig. 4.3). As a result, sirolimus blocks T-cell proliferation but its effects are downstream of the interleukin-2 receptors. IL-2 binding to its receptors activates intracellular protein kinases that in turn activate gene transcription and T-cell proliferation.

Sirolimus induces cytotoxicity resulting from the generation of reactive oxygen species (ROS) that regulate Bak protein expression and mitochondrial dysfunction. G2/M phase cell cycle arrest is the result of decreased expressions of CDK2 and cyclin B1. The rapamycin-receptor complex binds to mammalian target of rapamycin (mTOR) or FK506-binding protein-12-rapamycin-associated protein 1(FRAP1) that is encoded by the mTOR gene. Mammalian target of rapamycin (mTOR) is a serine threonine kinase and is a member of phosphatidylinositol-3-kinase (PI3K)-related kinases (PIKKS). mTOR is a downstream effector of PI3K/AKT pathway

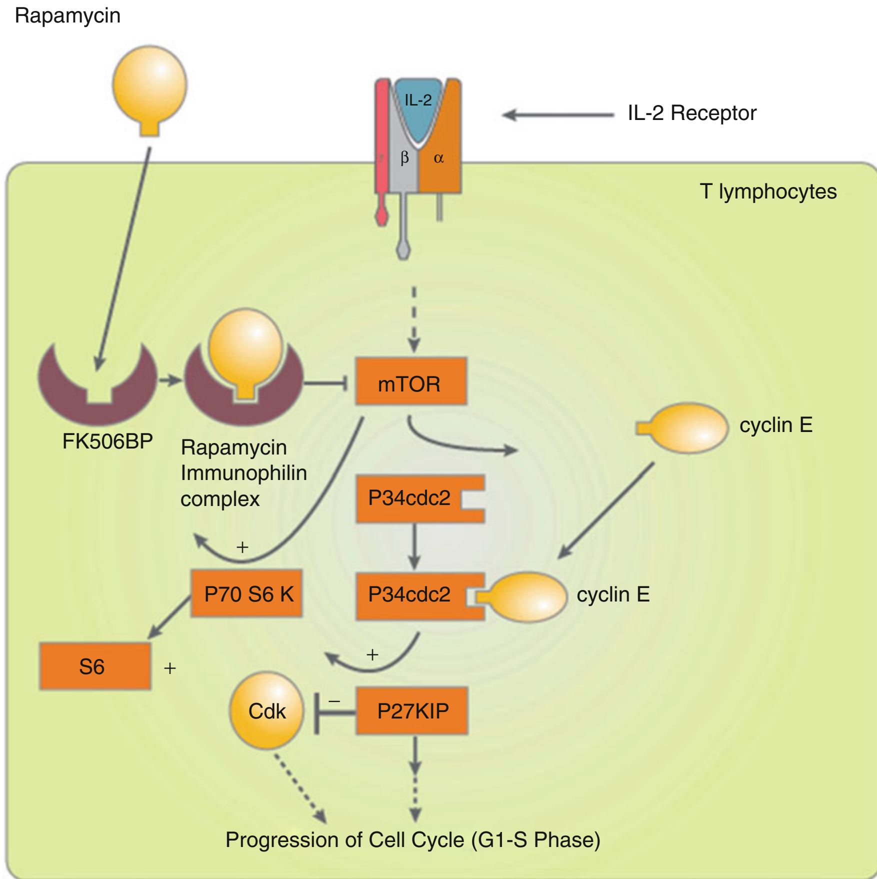

Fig. 4.3 The mechanism of action of sirolimus: sirolimus readily diffuses into the cytoplasm of the target cells where it binds to immunophilins (FK506-BP). The sirolimus-immunophilin complex does not inhibit calcineurin activity; instead it binds to the mammalian target of rapamycin (mTOR). The sirolimus-immunophilin-mTOR complex stops the cell cycle progression from G1 to S phase. The targets of sirolimus include the eukaryotic initiation factor (eIF-4 F), 70 KD S6 protein kinase (p70S6k), and several cyclin dependent kinases (cdk). As a consequence, it blocks downstream signaling pathway initiated after activation of IL-2 receptors resulting in blocking T-cell proliferation

and is a catalytic subunit of mTORC1 and mTORC2, which are two structurally distinct complexes. They are present in different subcellular compartments, where they regulate their function and activation. Rapamycin inhibits mTORC1 to exert its known effects. However, the effects of rapamycin on mTORC2 are more complex. mTORC2 signaling pathway is not yet very well defined. It inhibits the mTOR pathway by preventing the activation of mTORC1. The mTOR/PI3K/AKT pathway is an intracellular signaling pathway important in regulating the cell cycle. Rapamycin inhibits T-cell proliferation in response to various cytokines including IL-1, IL-2, IL-3, IL-4, IL-6, IGF, PDGF, and colony-stimulating factor.

4.3.1.2 Absorption, Distribution, and Excretion

Sirolimus is rapidly absorbed after it is given orally, and in healthy individuals, peak blood levels are achieved in about an hour after oral administration. However, it takes twice as long to reach peak blood levels in kidney transplant patients. Its systemic availability is about 15 % and high-fat meals interfere with the bioavailability. Sirolimus is bound (40 %) to proteins in plasma, and its elimination half-life is about 12–15 h for transplant patients but may vary. It is predominantly metabolized by CYP2A4; the drug has a number of active metabolites and is excreted in feces.

4.3.1.3 Drug Interactions

Since sirolimus is metabolized by CYP2A4 and is a substrate of the P-glycoprotein (P-GP) drug efflux pump, drugs such as voriconazole, itraconazole, fluconazole, and erythromycin increase its blood concentration. Conversely, the inducers of CYP3A4 will decrease blood levels of sirolimus. Cyclosporine increases the bioavailability of sirolimus, possibly due to P-GP inhibition and competition for CYP3A4. The bioavailability is more than 30–40 % when the two drugs are administered 4 h apart and more than 100 % when given at the same time. The combination of tacrolimus and sirolimus produced more renal toxicity than cyclosporine and sirolimus administered together.

4.3.1.4 Toxicity

The serious side effects of sirolimus may include an allergic reaction, increased risk of infection, and lymphoma. Nephrotoxicity is not a concern when the drug is not used in combination with cyclosporine or tacrolimus. Less serious side effects associated with the administration of sirolimus include upset stomach, increased cholesterol and triglyceride levels, acne, insomnia, tremor, sore or weak muscles, water retention or swelling, anemia, leukopenia, and thrombocytopenia. It could also impair wound healing.

4.3.1.5 Clinical Uses

Sirolimus is used for tissue transplantation where its major advantage over calcineurin inhibitors is that it is not nephrotoxic. Chronic renal failure in transplant patients who have taken calcineurin inhibitors long term can be prevented by the administration of sirolimus. Steroid-free immunosuppression can be achieved by administering sirolimus alone or in combination with mycophenolate mofetil and cyclosporine or tacrolimus. Since impaired wound healing is one of its potential side effects, some transplant centers only use sirolimus after several weeks of surgery.

Sirolimus has also been used in the production of sirolimus-eluting stents. These stents are used to treat obstructive coronary arteries. The rationale for the use of sirolimus in these stents is due to its antiproliferative activity. It has also received attention for cancer treatment due to its antiproliferative effects. In animal studies, sirolimus has shown some potential in the treatment of cancer where, in combination with doxorubicin, a remission for AKT-positive lymphomas has been observed.

4.3.2 Everolimus (Zortress)

Everolimus is a derivative of sirolimus and although its mechanism of action is similar to sirolimus, it is more hydrophilic and has a distinct pharmacokinetic profile. It has a shorter half-life than sirolimus and works well in cardiac allograft vasculopathy or posttransplant lymphoproliferative disorders. Everolimus binds to its receptor, FKBP12, in the cytoplasm, and the complex links to mTORC1, resulting in the inhibition of downstream signaling pathways. It acts through inhibition of mammalian target of rapamycin (mTOR) and is used to prevent organ rejection (Zortress) and some forms of cancer (Afinitor). Everolimus produces its response via binding to mTORC1 protein complex but has no effect on mTORC2. This results in the activation of AKT as it inhibits the negative feedback loop via inhibition of mTORC1. The ultimate effect is the inhibition of mRNA that codes for proteins involved in cell cycle, thus inhibiting the growth and proliferation of the target cells.

It is used in kidney, heart, liver, and other transplants. Everolimus reduces chronic allograft vasculopathy, which underlines its role in cardiac transplantation. Nonetheless, there is some controversy regarding its use in cardiac transplants due to increase death rate. It is also used as an immunosuppressant to prevent restenosis in drug-eluting coronary stents and is called the Promus everolimus-eluting coronary stent system.

Everolimus is usually administered with cyclosporine, and the nephrotoxicity by cyclosporine needs to be closely monitored depending on the dose, and it also produces hyperlipidemia. The common side effects of everolimus include increased risk of infection and certain forms of cancer, rash, edema, leukopenia, swelling of the face, arms, and legs, chest pain, difficulty in breathing and swallowing, flu-like symptoms, mouth sores, tingling of hands and feet, gastrointestinal disorders, changes in weight, and difficulty in urination. Within 30 days, everolimus may increase the risk of developing a blood clot that may damage the transplanted kidney. Everolimus is also a substrate for CYP3A4; as a result, the drugs affecting this enzyme system will alter its blood levels and clearance. Several drugs interact with everolimus including ACE inhibitors, statins, fibrates, azole antifungal agents, macrolide antibiotics, digoxin, dilitazem, verapamil, carbamazepine, dexamethasone, phenobarbital, rifampin, and protease inhibitors.

4.4 Sphingosine-1-Phosphate Receptor (S1P-R) Modulators

4.4.1 Fingolimod (Gilenya)

Fingolimod is a synthetic analogue of myriocin (ISP-1). It is derived from the culture filtrates of the fungus *Isaria sinclairii*. After phosphorylation, it binds to G-protein-linked sphingosine-1-phosphate receptor 1 (S1P1) on lymphocytes and thymocytes with high affinity, resulting in the internalization of the receptors. As a result, the cell is not able to respond to serum lipid sphingosine-1-phosphate (SIP), and due to the lack of this signal, they are unable to egress from lymphoid organs. Consequently, the recirculation of lymphocytes into graft site and peripheral inflammatory tissues is inhibited. Fingolimod has novel immunosuppressive mechanism of action as it induces a reduction in peripheral blood lymphocyte count through emigration of blood lymphocytes to the secondary lymphoid tissue as well as apoptotic T-cell death. The emigration of lymphocytes to the secondary lymphoid tissue is achieved through its modulation of the lymphocyte chemotactic response to chemokines that induces accelerated trafficking of T cells in the secondary lymphatic tissue (Fig. 4.4). It also acts on endothelial cells where fingolimod preserves vascular integrity as a result of augmenting endothelial barrier function and adherens junction assembly. Fingolimod does not affect activation, proliferation, and effector function of T and B cells. It has similar efficacy in prevention of acute graft rejection as mycophenolate mofetil in new renal transplant patients. However, patients switched from fingolimod to mycophenolate mofetil exhibit significant improvement in arterial vasodilatory function who were also receiving cyclosporine. It works in synergism with both the calcineurin inhibitors and the proliferation signal inhibitors to promote their immunosuppressive effects after tissue transplantation. Pharmacokinetics is characterized by a prolonged absorption phase, a large distribution volume, and a long elimination half-life. The most common side effect associated with fingolimod is asymptomatic transient bradycardia. It does not produce hyperlipidemia, diabetes mellitus, and renal toxicity, which are characteristic side effects of other immunosuppressive agents.

Fingolimod is the first oral drug approved for multiple sclerosis to decrease relapse and delay the progression of disability in patients, who are undergoing relapse. Multiple sclerosis is an inflammatory demyelinating and neurodegenerative disease of the central nervous system characterized by damage in insulating covers of the nerve cells, the brain, and the spinal cord. As a result, there is interference in the communication between neurons resulting in the symptoms of the disease. Immune system seems to play a role in multiple sclerosis, which is often characterized as an autoimmune disorder or a better term, immune-mediated disorder. The hallmark of the disease is the production of plaques accompanied by infiltration of T cells, B lymphocytes, plasma cells, and macrophages. Plaque production in multiple sclerosis is unique that differentiates it from other inflammatory diseases. T cells play a lead role in the inflammatory component of the disease. There is also an increase in the expression of Toll-like receptors. Autoreactive T cells are involved

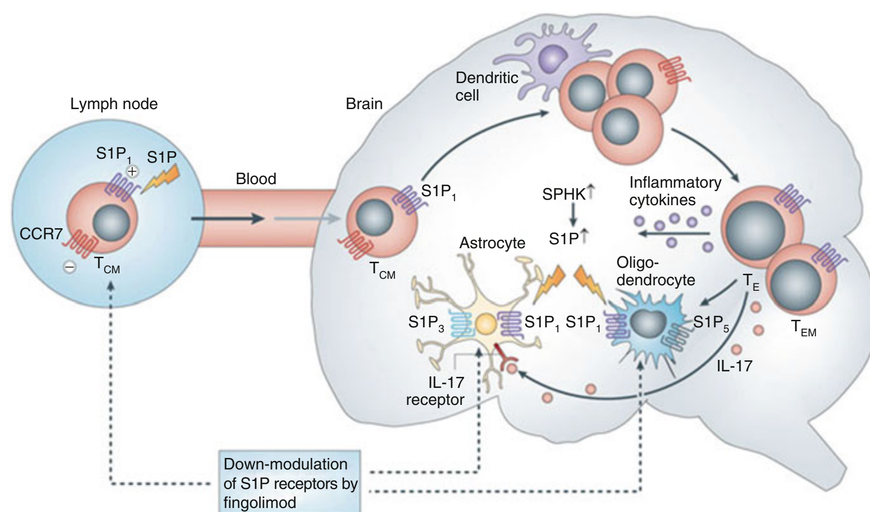

Fig. 4.4 The mechanism of action of fingolimod: fingolimod binds to G-protein linked sphingosine-1-phosphate receptor 1 (SIPI) on lymphocytes and thymocytes which results in the internalization of these receptors. As a result the cells are unable to respond to sphingosine 1 phosphate. As a consequence, lymphocytes cannot egress from the lymphoid organs. There is also a reduction in peripheral lymphocyte count through apoptotic cell death (Source: Brinkmann V et al (2010) Fingolimod: discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Dis* 9:883–897. Nature publishing group. With permission)

in the destruction of myelin sheaths. The inflammatory process after attack on myelin includes the release of various cytokines and production of antibodies. Activation of macrophages and the release of additional cytokines produce swelling and further destruction of the neurons. Fingolimod is able to sequester lymphocytes in the secondary lymphoid organs by inhibiting their egression. As a consequence, it prevents lymphocytes from getting to the central nervous system and causing the autoreactive responses and the resulting damage.

Fingolimod is being evaluated as a drug to treat heart failure and arrhythmias as well. Multiple signal pathways are associated with the pathogenesis of heart failure and arrhythmias. One of these pathways involves P21-activated kinase-1 (Pak1). P21-activated kinases are enzymes that are a family of serine/threonine protein kinases. They are associated with the G-protein signaling pathway activated by Cdc42 and Rac1. Cdc42 and Rac1 are GTPases, which upon induction cause the activation of P21-activated kinases. Three of its isoforms are expressed by the heart. Pak1 regulates many functions of the heart including cardiac cell growth, motility, and survival. Fingolimod activates Pak1 and therefore produces the cardioprotective effect. It prevents cardiac hypertrophy and arrhythmias in animal models.

4.5 Inhibitors of Costimulatory Molecules of T-Cell Activation

4.5.1 Belatacept (Nulojix)

Belatacept is a drug generated by recombinant DNA technology. It is composed of the fused Fc region of the antibody IgG1 to the extracellular domain of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is also called CD152. CTLA-4 is distributed on the surface of helper T cells and transmits inhibitory signals. Intracellular CTLA-4 is also present on regulatory T cells. In structure, CTLA-4 is similar to CD28 and binds to CD80/86 on antigen-presenting cells. CD28 and CD 80/86 are costimulatory molecules responsible for the activation of T cells. CTLA-4 disrupts this mechanism for which a couple of scenarios have been proposed. According to one suggestion, CTLA-4 may function by removing CD80 and CD86 from the cell surface of antigen-presenting cells. Alternatively, it may act by modulating the cell motility and cell signaling through PI3-kinase.

Belatacept binds to CD80/86 and prevents the second signal of the activation of helper T cells and even deactivates them. Without the second signal, T cells will not be activated and become anergic. CTLA-4 selectively blocks the process of T-cell activation (Fig. 4.5). It is intended to provide extended graft survival while limiting the toxicity generated by standard immune suppressing regimens, such as calcineurin inhibitors.

Belatacept in combination with basiliximab, mycophenolate mofetil [MMF], and corticosteroids is indicated for prophylaxis of organ rejection in adults receiving a kidney transplant. It should be given only to patients who are Epstein–Barr virus (EBV) seropositive. The patients receiving belatacept are at increased risk for developing posttransplant lymphoproliferative disorder (PTLD), predominantly involving the central nervous system (CNS). The other known adverse effects include cytomegalovirus (CMV) infection, T-cell-depleting therapy, and progressive multifocal leukoencephalopathy (PML). There is also increased risk of infection, including bacterial, viral, fungal, and protozoal infections, opportunistic infections and tuberculosis, and development of malignancies.

4.5.2 Abatacept (Orencia)

Abatacept is generated by recombinant DNA technology. It is composed of the fused Fc region of the antibody IgG1 to the extracellular domain of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 is distributed on the surface of helper T cells and transmits an inhibitory signal. In structure, CTLA-4 is similar to CD28 and binds to CD80/86 on antigen-presenting cells (Fig. 4.6). CD28 and CD80/86 are costimulatory molecules responsible for the activation of T cells. Abatacept differs from belatacept by two amino acids. It binds to CD80/86 and

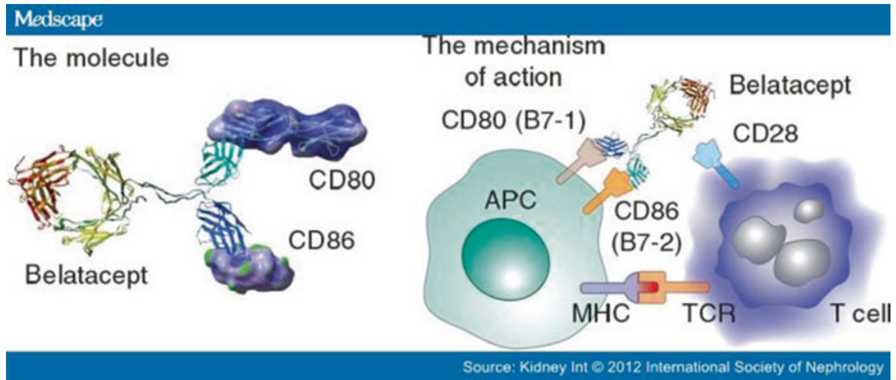

Fig. 4.5 The mechanism of action of belatacept: belatacept is an antagonist of the B7 ligands, CD80 (B7-1) and CD86 (B7-2), which are present on antigen presenting cells and are required for the initiation of second signal for the activation of T cells. Belatacept blocks the interaction between CD80/CD86 and CD28 (Source: Flavio V (2012) Are calcineurin inhibitors – free regimens ready for prime time? *Kidney Int* 28:1054–1060. Nature publishing group. Reproduced with permission)

prevents the second signal for the activation of helper T cells and even deactivates them. Without the second signal, T cells will not be activated and may become anergic. It is used in patients, adults, and children, at least 6 years old to treat rheumatoid arthritis, who do not respond to other disease-modifying antirheumatic drugs. Abatacept delays the progression of structural damage and reduces the symptoms associated with rheumatoid arthritis. It should not be used in combination with Kineret and other drugs targeting TNF-α. The side effects may include fatigue, sore throat, dry cough, trouble breathing, wheezing, fever, chills, night sweats, flu symptoms, weight loss, skin irritation, and gastrointestinal discomfort.

4.6 Cytotoxic Agents

4.6.1 Mycophenolate Mofetil (Cellcept)

Mycophenolic acid (MPA) was isolated from cultures of *Penicillium* spp. in 1896 and purified in 1913. Initially, the compound was studied for its antifungal and antibacterial effects and later for its antitumor effects. Many years later, its immunosuppressive activities were recognized, and after further developmental work, an ester prodrug mycophenolate mofetil was developed that was approved by the United States Food and Drug Administration for the prevention of acute renal allograft rejection in 1995 and for heart transplant recipients in 1998. Mycophenolate mofetil is a cytotoxic agent now used for immunosuppressive therapy and is the mofetil ester of mycophenolic acid that is the active immunosuppressive agent.

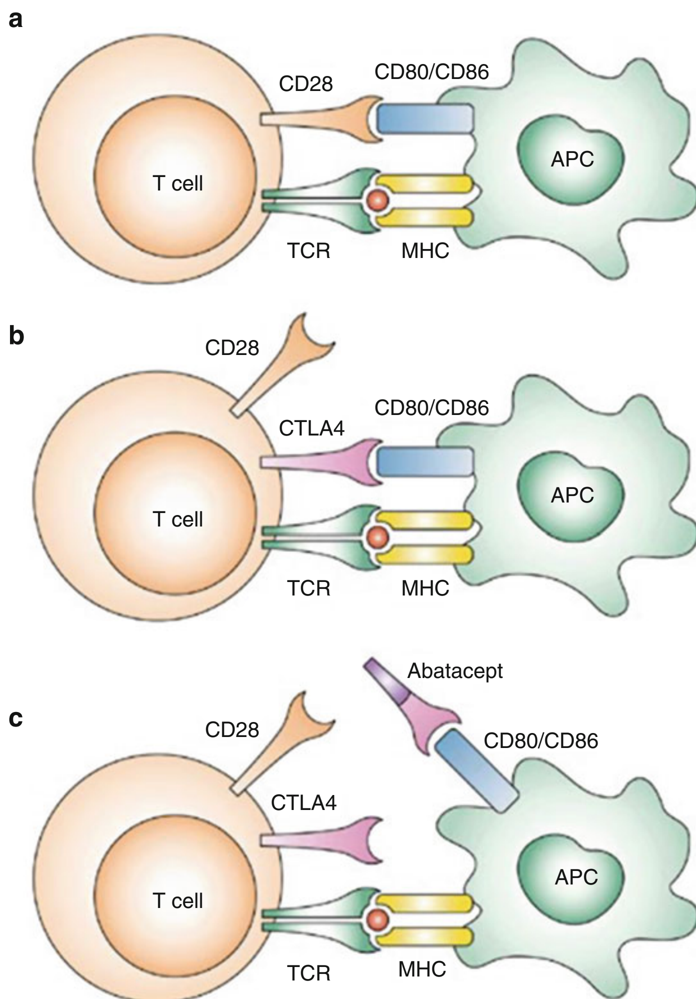

Fig. 4.6 The mechanism of action of abatacept: abatacept is an antagonist of the B7 ligands, CD80 (B7-1) and CD86 (B7-2), which are present on antigen presenting cells and are required for the initiation of second signal for the activation of T cells. Following activation of T cells there is an increase in the expression of CTLA-4. CTL4 binds to CD80/86 and suppresses T cell activation. Abatacept blocks the interaction between CD80/CD86 and CD28 (Reproduced with permission. Source: Ruderman EM, Pope RM (2006) Drug insight: abatacept for the treatment of rheumatoid arthritis. *Nature Clin Pract Rheumatol* 2: 654–660. Nature Publishing Group, reproduced with permission)

4.6.1.1 Mechanism of Action

Mycophenolate mofetil is a functionally selective cytotoxic agent for B and T lymphocytes, where it blocks the production of guanosine nucleotides required for DNA synthesis. For purine biosynthesis, B and T lymphocytes rely on the de novo synthesis rather than the salvage pathway. Lymphocytes have little or no salvage

pathway as opposed to other blood marrow elements and parenchymal cells that depend on salvage pathways for the production of guanosine nucleotides. Mycophenolate mofetil is a selective, noncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase (IMP-DH), which is associated with the de novo synthesis of guanosine nucleotides. As a result, it suppresses lymphocyte proliferation and antibody synthesis by B lymphocytes. Furthermore, mycophenolate mofetil may suppress the recruitment of leukocytes to the sites of inflammation by deglycosylating lymphocytes glycoprotein involved in adhesion and cell migration, which could be useful in organ transplantation. The synthesis of cytokines and their receptors and cytokine receptor-dependent signal transduction mechanisms are not specifically affected by mycophenolate mofetil.

4.6.1.2 Absorption, Distribution, and Excretion

Mycophenolate mofetil is rapidly absorbed after oral administration, and the bioavailability of its oral dose is 94 %. It is metabolized by esterases to free mycophenolic acid, which is the active metabolite. The enterohepatic recirculation plays a crucial role in the serum levels of MPA. The active metabolite is further metabolized by glucuronyl transferase and is eliminated (90 %) in urine as the MPA glucuronide as a result of organic anion transport system in the proximal tubule. A small amount is excreted in feces.

4.6.1.3 Drug Interactions

The coadministration of mycophenolate mofetil with antacids results in decreased absorption. The plasma MPA concentration is significantly reduced by cholestyramine due to binding of the cholestyramine to MPAG in the intestine and interfering with the enterohepatic recirculation of the drug. The bioavailability of mycophenolate mofetil is higher when administered with tacrolimus as opposed to cyclosporine. The bioavailability of MPA is reduced by antibiotics including fluoroquinolones and metronidazole.

4.6.1.4 Toxicity

The side effects of mycophenolate mofetil include diarrhea, abdominal pain, constipation, nausea/vomiting, acne, dyspnea, cough, peripheral edema, increased risk of infections, drug-induced fever, dizziness, headaches, leukopenia, and anemia.

4.6.1.5 Clinical Uses

Mycophenolate mofetil is used for tissue transplantation in combination with tacrolimus or cyclosporine or sirolimus plus glucocorticoids. It is used more than any other cytotoxic drug either at the time of the transplant or following the initiation of

acute rejection. Mycophenolate mofetil is a prophylactic agent and cannot be used for chronic rejection or ongoing acute rejection.

4.6.2 Azathioprine

Originally developed for chemotherapy, azathioprine is used today mainly as an immunosuppressive agent and rarely as an antineoplastic drug. It was introduced as an immunosuppressive agent by a British pioneer of tissue transplantation, Roy Calne. Azathioprine is used to prevent rejection after tissue transplantation for tissue transplants as a replacement for 6-mercaptopurine because it is less toxic. In addition to tissue transplantation, it is also used for rheumatoid arthritis and Crohn's disease. Azathioprine is a prodrug that in the body is converted to its active metabolites 6-mercaptopruine and 6-thioinosinic acid. Until the discovery of cyclosporine, azathioprine in combination with steroids was the standard treatment to prevent rejection after tissue transplantation.

4.6.2.1 Mechanism of Action

Azathioprine inhibits purine synthesis, which is necessary for the proliferation of cells, especially immunocompetent cells. It is converted to 6-mercaptopurine after it reacts with glutathione, and its metabolite, 6-mercaptopurine, is converted to additional metabolites that inhibit de novo purine synthesis. This results from the synthesis of 6-thio IMP, 6-thio GMP, and 6-thio GTP, and cell proliferation is inhibited after 6-thio GTP gets inserted into host DNA.

4.6.2.2 Absorption, Distribution, and Excretion

Azathioprine is orally absorbed with maximum blood levels attained within 1–2 h. The half-life of the parent drug is 10 min, but its metabolite, 6-mercaptopurine, has a half-life of nearly 1 h, and some metabolites have longer half-life. The prodrug and the active metabolites both bind to plasma proteins with low affinity and are removed from tissues by oxidation or methylation.

4.6.2.3 Drug Interactions

The administration of purine analogues such as allopurinol and azathioprine together is not recommended. The enzyme thiopurine S-methyltransferase (TPMT) inhibits the activity of 6-mercaptopurine. Genetic polymorphisms of TPMT could increase azathioprine toxicity, and therefore, measuring levels of serum TPMT will

be helpful in avoiding this toxic effect. Leukopenia, anemia, and thrombocytopenia could develop when azathioprine is administered in conjunction with angiotensin-converting enzyme inhibitors or other drugs, which cause myelosuppression.

4.6.2.4 Toxic Effects

Azathioprine is considered as a human carcinogen although some of these studies have been suggested to be inconclusive. Individuals who have been previously treated with alkylating agents may be at a higher risk for cancer when treated with azathioprine. It is not known to cause fetal malformation. The short-time adverse effects associated with the use of azathioprine include myelosuppression including anemia, leukopenia, and thrombocytopenia. Other side effects include increased risk of cancer and infection, G.I. disturbances, alopecia, and hepatotoxicity.

4.6.2.5 Clinical Uses

Azathioprine is administered to patients who do not respond to calcineurin inhibitors, sirolimus, and glucocorticoids. Daily doses of 3–10 mg/kg of azathioprine are administered 1 or 2 days before renal transplantation or on the day of surgery for prophylactic therapy. Mycophenolate mofetil is increasingly used in place of azathioprine for tissue transplantation since it is less myelotoxic and causes few opportunistic infections.

4.6.3 Cyclophosphamide

Cyclophosphamide disturbs the mechanisms associated with DNA synthesis and cell proliferation by alkylating DNA in proliferating and nonproliferating cells. Its mechanisms of immunosuppressive effects are similar to its antineoplastic actions. Cyclophosphamide affects both B and T cells, but it produces more toxicity to B cells because they recover slowly. It has some unpredictable effects on T-cell-mediated immunity where it actually augments some T-cell-mediated responses; however, the overall response is inhibitory.

Most of the preparative regimens used prior to allogeneic bone marrow transplantation, to avoid the risk of rejection, include cyclophosphamide in combination with another cytotoxic agent ± antithymocyte globulin or total lymphoid irradiation. The cytotoxic agents used in addition to cyclophosphamide are busulfan, fludarabine, or treosulfan. A combination of fludarabine and cyclophosphamide ± antithymocyte globulin has also been used as preparative regimens for cord blood transplantation and allogeneic stem cell transplantation to avoid the risk of rejection.

4.7 Glucocorticoids

Cortisone was the first immunosuppressant identified. Glucocorticoids are steroids synthesized by the adrenal cortex and are made up of 21 carbon atoms. While they possess multiple biological functions affecting numerous tissues and organ systems, only their immunosuppressive effects will be discussed here. Since the 1960s, glucocorticoids have been used in tissue transplantation to prevent rejection. Today, they are used in combination with other immunosuppressive agents to prevent rejection of the transplanted tissue. However, due to their serious effects, the focus has been on glucocorticoid withdrawal soon after tissue transplantation and switching to steroid-free immunosuppressive regimens.

4.7.1 Mechanism of Action

Glucocorticoids inhibit acquired or cell-mediated immunity. Their effects are mediated via inhibition of genes that code for a variety of cytokines. Glucocorticoids inhibit the production of most cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and INF-γ. IL-2 inhibition by corticosteroids is the most crucial effect in immunosuppression, which results in the inhibition of T-cell proliferation and activation of cytolytic T cells. Glucocorticoids also slightly affect humoral immunity by inhibiting B-cell clonal expansion and antibody synthesis, and these effects are mediated via their ability to inhibit B cells ability to express IL-2 and IL-2 receptors.

Glucocorticoids are potent inhibitors of all phases of the inflammatory process. This is accomplished by their induction of lipocortin (annexin-1) synthesis. Lipocortin binds to the cell membrane and prevents the access of phospholipase A_2 to its substrate arachidonic acid, resulting in diminished eicosanoid production. This effect is further pronounced by the inhibition of cyclooxygenase. Furthermore, lipocortin 1 escaping to the extracellular space is also stimulated by glucocorticoids, which subsequently cause inhibition of inflammation by binding to various leukocyte membrane receptors. The inflammatory processes affected by this mechanism include: adhesion, chemotaxis, respiratory burst, and phagocytosis. It also inhibits the release of various inflammatory mediators from mononuclear and polymorphonuclear phagocytes. Glucocorticoid-receptor complex inhibits NF-κB via increased expression of IκB, which results in increased apoptosis of activated cells. Overall, glucocorticoids cause a rapid, transient decrease in the number of circulating peripheral blood lymphocytes.

4.7.2 Absorption, Distribution, and Excretion

Glucocorticoids are administered orally, intravenously, or intramuscularly. They are absorbed locally at the site of administration including the skin, eye, and respiratory tract. Prolonged local exposure could eventually produce systemic effects. Most of the

corticosteroids are bound to two plasma proteins, transcortin (corticosteroid-binding globulin, CBG) and albumin. At higher doses, when all the plasma-binding protein sites are saturated, corticosteroids could exist in unbound form. Their metabolism results in the formation of water-soluble derivatives. Their reduction can take place both in the liver and outside the liver, resulting in inactive compound, and subsequent reduction is only hepatic. These enzymatic reactions convert glucocorticoids either into glucuronide or sulfate form, which are then excreted in urine.

4.7.3 Side Effects

The most common side effects of glucocorticoids when used to prevent transplant rejection include hyperglycemia, increased risk of infection, poor wound healing, bone loss, ulcers, muscle weakness, water retention, skin irritation, excess facial hair growth, and facial puffiness. In children, their use could also cause growth retardation.

4.7.4 Clinical Uses

The glucocorticoids are employed in combination with other immunosuppressive agents to prevent transplant rejection. For acute transplant rejection, high doses of intravenous methylprednisolone are used. They are useful in suppressing allergic reactions to other immunosuppressive agents as well as the effects of cytokines associated with the use of anti-CD3. The glucocorticoids are used for bone marrow transplantation to prevent graft-versus-host disease. They are also used to treat autoimmune diseases, asthma, psoriasis, dermatomyositis, and inflammatory manifestations of the eye.

4.8 Polyclonal Antibodies

4.8.1 Antithymocyte and Antilymphocyte Globulins

Antithymocyte and antilymphocyte globulins are generated in animals against human T cells and are used for acute rejection in organ transplantation and aplastic anemia. These polyclonal antibodies also possess cytotoxic antibodies that bind to a variety of antigenic markers on T lymphocytes, resulting in their depletion from the circulation. Furthermore, they inhibit lymphocyte function by binding to important lymphocyte regulatory molecules on lymphocyte cell surface.

Two antithymocyte globulins are approved for use in the United States. Atgam is an equine immunoglobulin and thymoglobulin is a rabbit immunoglobulin. Antithymocyte globulins are used in combination with other immunosuppressive

agents for the treatment of acute renal transplant rejection. They may be used initially in renal transplant patients instead of calcineurin inhibitors, which are nephrotoxic, to protect the transplanted tissue. Antithymocyte globulins are used at a dose of 1.5 mg/Kg per day for 1–2 weeks for acute rejection of renal grafts. They have also been used for liver transplantation. It is also administered at the time of transplant to prevent graft-versus-host disease in the United States, while in Europe it is preferred for steroid-resistant acute rejection. However, both preparations are also used, before or during the kidney transplant as off-label applications. The side effects of antithymocyte globulin include fever, chills, serum sickness, leukopenia, thrombocytopenia, increased risk of infection, and malignancies when used in combination with other immunosuppressive drugs. Since there is increased risk of infection and neoplasm, there is a controversy regarding their use, questioning whether benefits outweigh the risks.

Antilymphocyte globulins (ALG) are less used than antithymocyte globulins and do not have a brand name. The source of the antibody is mostly equine. Both carry the risk of producing posttransplant lymphoproliferative disorder and cytokine-release syndrome. Posttransplant lymphoproliferative disorder is B-cell proliferation due to therapeutic immunosuppression. The uncontrolled proliferation of B cells follows infection with Epstein–Barr virus and can be treated with cyclosporine or tacrolimus. Some of these B cells may undergo mutation and develop cancer (frank lymphoma). Cytokine-release syndrome, another complication of this therapy, is produced when antibodies to T cells bind to T cells and activate them before they are killed. This results in the production of a variety of cytokines secreted by T cells, producing a systemic inflammatory response. It causes hypotension, fever, and shivering and is similar to an infection. The condition can be treated by corticosteroids, antihistamines, and acetaminophen.

4.8.2 Rho (D) Immune Globulin

Rho (D) immune globulin is one of the most specific and effective immunosuppressive treatments available. These IgG antibodies have high Rh (D)-specific titers. Administration of Rho (D) immune globulin prevents the response, which develops in Rh^- mothers who were pregnant with Rh^+ fetus and consequently have become sensitized to the D antigen on fetal erythrocytes of the infant. In these Rh^- mothers, the antibody titers against Rh^+ cells will continue to rise after each subsequent pregnancy, resulting in hemolytic disease of the newborn and erythroblastosis fetalis. This disease can be prevented by the administration of Rho (D) immunoglobulin to the Rh^- mother within 72 h of the birth of an Rh^+ baby. The antibody could also be given after miscarriage, abortion or ectopic pregnancy. Rho (D) immune globulin is administered intramuscularly and its half-life is about 3–4 weeks. The side effects include discomfort at the site of injection and mild fever. Another very rare side effect is anaphylactic shock.

4.9 Monoclonal Antibodies

The monoclonal antibodies used as immunosuppressive agents in tissue transplantation include muromonab-CD3, daclizumab and basiliximab. Muromonab-CD3 binds to a specific site on CD3 receptor and interferes with the ability of the TCR to the antigen and also inhibits CD3 receptor-dependent signal transduction mechanisms, all of which result in immune suppression. Both daclizumab and basiliximab are monoclonal antibodies directed against IL-2 receptors and consequently inhibit IL-2-dependent responses after tissue transplantation, resulting in immune suppression. The monoclonal antibodies used as immunosuppressive agents are described in detail in Chap. 5.

4.10 Future Directions

The development of novel immunosuppressive agents in the past two decades has resulted in a reduction in the incidence of acute rejection following tissue transplantation. However, the non-immune side effects of the maintenance immunosuppressive regimens have hindered the successful long-term outcomes. Consequently, a more selective group of immunosuppressive therapeutic agents is required that will not target ubiquitously expressed receptors and thus will have far fewer undesired side effects, which continues to be a major challenge for the available compounds.

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Chapter 5
Monoclonal Antibodies as Therapeutic Agents

Abstract Monoclonal antibodies are effective targeted therapeutic agents. The high specificity of the antibodies makes them ideal to reach their intended target and thus is useful to treat a variety of disease states. This chapter begins with an explanation of methods which are used to propagate and isolate monoclonal antibodies. There is description of hybridoma technology, CDR grafting, and phage display technology. These techniques are used to develop various therapeutic monoclonal antibodies, including chimeric, humanized, and human monoclonal antibodies. This is followed by the description of specific monoclonal antibodies used as drugs to treat various diseases including autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, and multiple sclerosis), immune system-mediated disorders (psoriasis), transplant rejection, thrombosis, cancer, allergic disease, as well as others. Immunoconjugates which are comprised of monoclonal antibody linked to a chemotherapeutic agent or source(s) of radiation are discussed to treat cancer. The treatment of cancer includes both hematologic malignancies as well as solid tumors. The target of each monoclonal antibody is identified along with its precise mechanism of action, clinical use, and adverse effects.

Keywords Hybridoma technology • Cloning • Chimeric antibodies • Humanized antibodies • CDR grafting • Phage display technology • Human antibodies • Fc fragment • Fab fragment • CDR-grafted antibodies (CDR) • Muromonab-CD3 binds • CD3 glycoprotein Σ chain • Daclizumab • Basiliximab • IL-2α receptor • Ustekinumab • p40 • IL-12 • IL-23 • TH17 cells • IL-17 • IL-6 • Secukinumab • Infliximab • Adalimumab • Golimumab • Tocilizumab • Certolizumab • Vedolizumab • Belimumab • B-cell activating factor (BAFF) • SLE • Natalizumab • α-4 integrin • α4β7 integrin • MADCAM1 (addressin) • Crohn's disease • Multiple sclerosis • Canakinumab • Cryopyrin-associated periodic syndromes (CAPS) • IL-1β • Abciximab • GPIIb/IIIa receptors • Rituximab • CD20 • Ofatumumab • Obinutuzumab • Trastuzumab • HER-2 • Pertuzumab • Alemtuzumab • CD52 • Cetuximab • Human growth factor • Human growth factor epidermal receptor (EGFR) • KRAS • Panitumumab • NRAS • Bevacizumab • Vascular endothelial growth factor • Ipilimumab • Malignant melanoma • Pembrolizumab • Programmed cell death 1

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receptor • PDL-1 • PDL-2 • Nivolumab • Trastuzumab emtansine • Gemtuzumab-ozogamicin • CD33 • Ibritumomab • Indium 11 • Yttrium-90 • Immunoconjugate • Tositumomab • Iodine-131 • Relapsed lymphoma • Refractory lymphoma • Low-grade lymphoma • Follicular lymphoma • Transformed non-Hodgkin's lymphoma • Brentuximab vedotin • Tumor necrosis factor receptor superfamily • TRAF1 • TRAF2 • TRAF3 • TRAF3 • TRAF5 • Eculizumab • Compliment component 5 • Paroxysmal nocturnal hemoglobinuria • Denosumab • RANKL • TRANCE • Ranibizumab • Omalizumab • IgE • Psoriasis • Breast cancer • Colorectal cancer • Ventricular dysfunction • Antibody-dependent cell-mediated cytotoxicity • Complement-dependent cell-mediated cytotoxicity

5.1 Introduction

In 1895, the first clinical trials were reported by Hericourt and Richet where they employed the principal of antibody production. They injected cancer cells into animals to obtain antiserum to treat cancer patients; this was the first example where several patients with cancer were administered tailor-made serum for treatment. Several patients showed improvement which was encouraging, but none of the patients were completely cured. These trials were again repeated in early 1900s, but the results were not consistent. The problems included the variability of the antisera and side effects of polyclonal antibodies – some of which were directed against self.

Paul Ehrlich, at the beginning of the twentieth century, first proposed the concept of the "magic bullets." He reasoned that the development of compounds that selectively target a disease-causing organism could potentially deliver toxins to that organism. The specificity of antibodies, which are extremely specific and bind to and attack one particular antigen, provided a powerful tool to advance the idea envisioned by Ehrlich. As a result, the concept that antibodies could be used as therapeutic agents was not far-fetched. His vision is being pursued today since antibodies are not only specific with high affinity but could also recruit effector functions of immune response. These effector mechanisms include antibody-mediated cytolysis, complement-mediated cytolysis, and antibody-dependent cell-mediated cytotoxicity. Furthermore, antibodies can be used to deliver radiation, chemotherapeutic agents, and/or toxins that make them extremely useful in treating infectious diseases, cancer, tissue rejection, graft vs. host disease, and autoimmune and inflammatory diseases.

Antibodies have been used for years to detect small levels of antigens in biological fluids. The focus of interest included enzymes, hormones, microorganisms, toxins, drugs, and other proteins. Their specificity provided a distinct advantage in targeting the intended antigen. However, their use in a diagnostic laboratory was limited due to severe problems with their propagation in large amounts. The specific sera could only be propagated by conventional immunization, which was further complicated by other issues such as inability to predict immune response, heterogeneity of specific antibodies, and immunogenicity of minor contaminants. This technique only produced polyclonal antibodies that possessed many different variable

regions. Furthermore, it was almost impossible to produce antibodies against weak and/or impure antigens.

Historically, antibodies have been obtained from the serum of animals. The serum contains a mixture of polyclonal antibodies. In 1890, Emil Behring immunized rabbits and mice against tetanus and diphtheria and reported that the antitoxin serum could protect against a lethal dose of the toxin. Since then, antisera have been used to protect from pathogens and toxins, but serum sickness had been a major drawback for their clinical use. Antisera may produce immune responses which could cause severe allergic reactions and may even lead to anaphylactic shock and death.

5.2 Production of Monoclonal Antibodies

In the 1970s, the B-cell cancer myeloma was known and it was established that these cells produced a single type of antibody. A revolution started in 1975, in the area of generating antibodies when Kohler and Milstein succeeded in fusing murine myeloma cells with sheep red blood cell-immunized splenocytes. This technique resulted in the production of purified antibodies and was named the hybridoma technology. This turned out to be an ideal technique to produce antibodies since each hybrid cell is made up of genetic material from a single myeloma cell, and a single B-cell produces only one type of antibody called a monoclonal antibody. This fused cell could be potentially propagated indefinitely, overcoming the problem of the limited production of antibodies and their heterogeneity.

To produce monoclonal antibodies by hybridoma technology, mice (or other animals) are injected with the antigen. After 72 h, spleens are removed and splenocyte suspensions are prepared; the splenocytes are then mixed with mouse myeloma cells grown in cultures. A chemical reagent or a virus is added in the mixture which causes the fusion of the cell membranes of splenocytes and myeloma cells. This results in the formation of random fused cells between splenocytes–myeloma cells, splenocytes–splenocytes, and myeloma cells–myeloma cells. Selection is performed via culturing the newly fused hybridoma cells in special media containing HAT (hypoxanthine–aminopterin–thymidine) medium for 10–14 days. Aminopterin in the medium blocks the de novo pathway of purine biosynthesis, which causes the death of unfused myeloma cells, since they are unable to produce nucleotides by de novo or salvage pathway. Under these conditions, only the hybrids of splenocyte–myeloma cells can survive and propagate, and the unfused myeloma cells and splenocytes will die. The surviving hybridomas are then cultured in small colonies (Fig. 5.1).

5.3 Screening and Cloning

The hybridomas are allowed to propagate 2–3 weeks. The screening of the desired hybridomas which are the hybrids producing the desired antibody is done by radioimmunoassay, enzyme-linked immunosorbent assay, or by flow cytometry. The desired

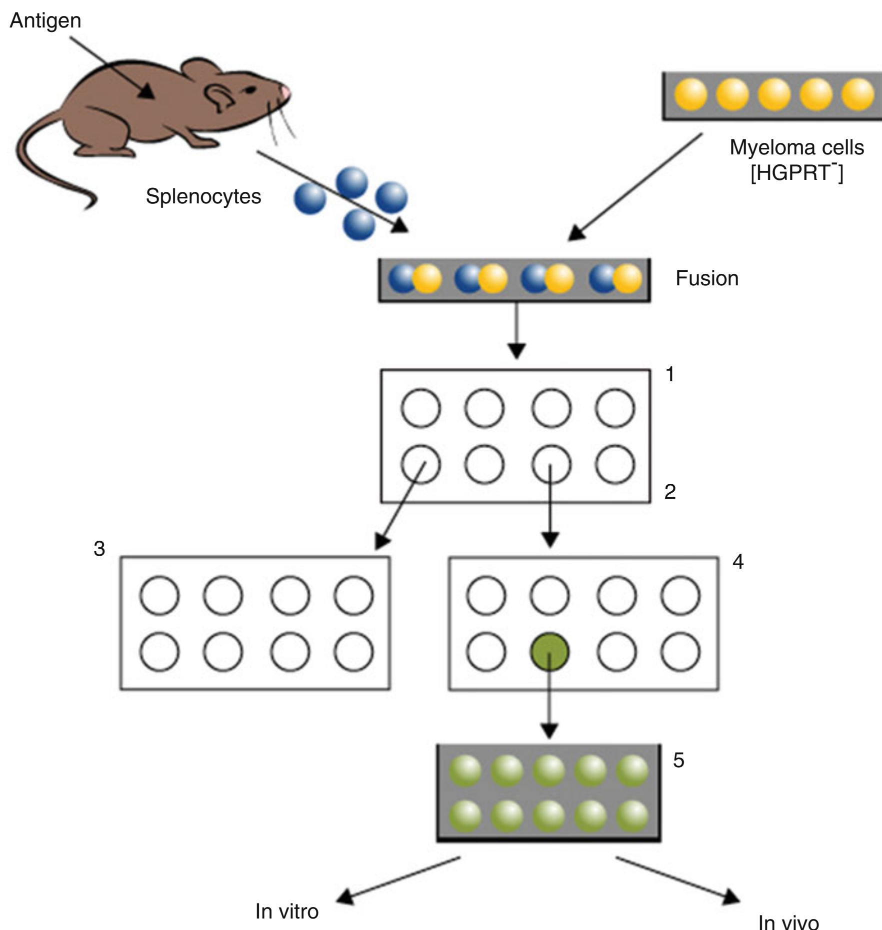


Fig. 5.1 Technique to produce monoclonal antibodies: After mice are injected with the antigen, the splenocytes are isolated and fused with myeloma cells. The fused cells are cultured (1) and the supernatants are assayed for the presence of desired antibodies (2). The cells from antibody-positive wells are cloned (3) and supernatants are tested for the desired antibodies (4) followed by expansion of the positive clones (5) and in vitro or in vivo propagation to produce the needed amount of monoclonal antibodies

hybrids are then cloned to ascertain the hybrids producing the desired antibody, are solely kept, and the rest are discarded. Various hybridomas in culture may secrete a mixture of antibodies directed against various epitopes on the antigen. The cloning is done by limited dilution where hybridomas are diluted and one cell is pipetted into a microtiter plate well. The single cell produces a colony of identical cells. A large-scale production of monoclonal antibodies is done by one of the many techniques, including tissue culture, injection into peritoneal cavities of mice, or industrial bioreactors.

5.4 Murine Antibodies as Therapeutic Agents

Following the development of hybridoma technology, the potential of using these antibodies appeared endless. However, when the mouse antibodies were employed clinically, quickly they were found to be of rather limited use. These murine antibodies were rapidly inactivated by human immune response because of the production of antibodies against the murine monoclonal antibodies. This reaction by the human immune response against mouse antibodies is called HAMA (human anti-mouse antibodies). This response not only produces flu-like symptoms, allergic reaction, and in extreme cases systemic shock or death, but also results in the rapid neutralization and clearance of mouse monoclonal antibodies being administered as therapeutic agent.

There have been advances, not only in the area of hybridoma and monoclonal antibody production but also in recombinant DNA technology. New information about the organization and expression of immunoglobulin genes in B cells as well as the rearrangement and mutation of germline immunoglobulin genes, which form the repertoire of functional immunoglobulin genes emerged. This resulted in combining hybridoma and recombinant DNA technologies to produce antibodies possessing more human components. As a consequence, recombinant antibodies have become important therapeutic agents, since they are designer antibodies that are less immunogenic, smaller, and have greater affinity for the target. Furthermore, they also possess the potential to carry more complex molecules including the radiation, toxins, chemotherapeutic agents, or enzymes.

5.5 Chimeric Antibodies

The word chimeric comes from chimera, a mythical beast with the head of a lion, the body of a goat, and the tail of a dragon. To overcome the problems associated with the HAMA response, the idea for chimeric antibodies was an important alternative. Their rationale was based on the ability of the Fc fragment of the antibody to conduct signal transduction mechanisms, where the fusion of Fc fragment of human with the FAB fragment from another species may result in minimizing the development of HAMA. By using the recombinant DNA technology, parts of the FAB fragment of murine antibody were added to the Fc fragment of the human antibody, and murine variable regions genes were cloned into a mammalian expression system. This contained human heavy and light chain constant region gene components, and the desired biological function led the way in choosing the Fc segment. As a result, this antibody has the binding characteristics of the mouse antibody and the signal transduction capabilities of the human antibody. The chimeric antibodies are 30 % mouse and 70 % human, and therefore they could still produce the HAMA response, referred to as human anti-chimeric antibody (HACA) responses. Human IgG_1 is the antibody of choice for chimeric antibodies when effector function is required as well. Otherwise, if the effector function of IgG_1 is not desired, other subclasses of IgG could be employed (Fig. 5.2).

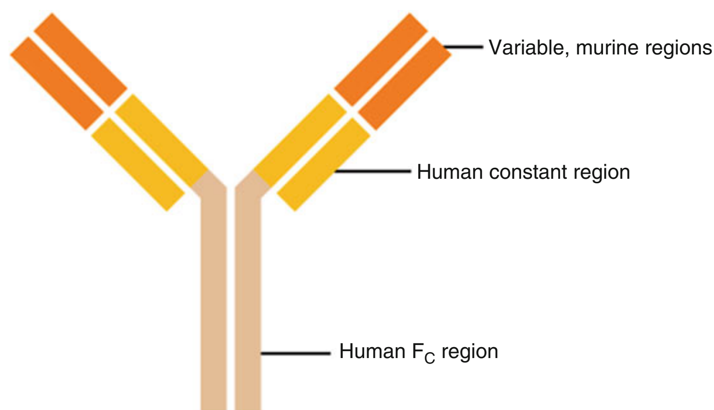


Fig. 5.2 Depiction of a chimeric monoclonal antibody

5.6 Humanized Antibodies

Humanized antibodies or complementarity-determining region (CDR)-grafted antibodies are produced by recombinant DNA technology. This technique was developed by Greg Winter and his colleagues in 1986 whereby only a part of the mouse antibody that binds to its target is inserted into human antibody. Also termed as second-generation or hyperchimeric antibodies, this technique employed CDR of the mouse antibody and is grafted into human antibody. The DNA encoding the CDR from the heavy and light chains is used. This technique produces an antibody that is more human than the chimeric antibody described in the previous section. The method was further improved by modifying any framework or "scaffold" residues that are important for the antibody binding site. These antibodies are now called as humanized antibody, which are 90–95 % human. However, human immune response could still react against the mouse region of the antibody.

5.7 Human Antibodies

Phage display technology or transgenic mice are used to produce 100 % human monoclonal antibodies. This is done by transferring human immunoglobulin genes into the mouse genome, and the transgenic mice are vaccinated with the antigen of choice. The ultimate result is the production of desired antibodies. This is in vitro transformation of mouse antibodies into fully human antibodies. Immune response against the monoclonal antibody may still be produced due to human IgG allotype. The stems for various types of monoclonal antibodies are shown in Fig. 5.3.

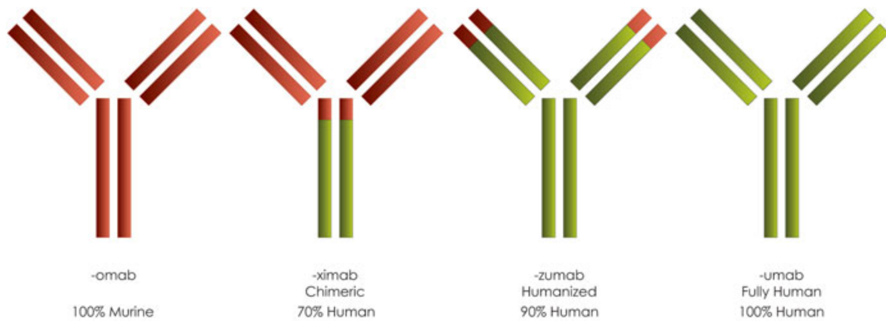


Fig. 5.3 Stems of monoclonal antibody nomenclature

5.8 Therapeutic Uses of Monoclonal Antibodies

5.8.1 Tissue Transplantation

5.8.1.1 Muromonab-CD3

Muromonab-CD3 binds to CD3 glycoprotein Σ chain that is in close proximity to the antigen recognition complex. The components of antigen recognition complex are involved in the recognition of the antigen, signal transduction, and proliferation of T lymphocytes. When muromonab-CD3 binds to the Σ chain of CD3, the antigen is blocked and is unable to bind to the TCR (Fig. 5.4). The inability of the TCR to bind to the antigen also results from its internalization following treatment with the antibody. It also binds to T cells resulting in their depletion from the blood and peripheral lymphoid organs, rapidly after its administration. Two to 7 days following the initiation of therapy, the numbers of $CD4^+$ and $CD8^+$ begin to increase in the peripheral blood without any consequence on the immunosuppressive effects of the antibody. The number of $CD3^+$ cells return to normal within a week after the end of therapy with the antibody. During the second week of therapy, there is a production of antibodies directed against the drug. The antibodies compete with the drug for binding to its receptors, and the antibody titer (>=1:1,000) must be monitored to decide if the treatment should be continued. The antibody also inhibits the secretion of a number of cytokines except IL-4 and IL-10. The effects on IL-2 secretion are particularly pronounced.

Muromonab-CD3 is used for the treatment of acute organ transplant rejection. It is effective in preventing graft rejection after kidney, heart, or liver transplantation. Muromonab-CD3 is effective in patients who, after acute cardiac or liver allograft rejection, do not respond to steroid therapy. It is administered intravenously, and with a dose of 5 mg/day, a general concentration range of 400–1,500 ng/ml can be achieved. A serum concentration of 600–1,150 ng/ml in renal transplant patients produces desirable immunosuppressive effects. The levels of CD3 expression, their production, and antibodies to the drug determine its rate of clearance. In the absence of antibodies to muromonab-CD3, its half-life is about 18 h.

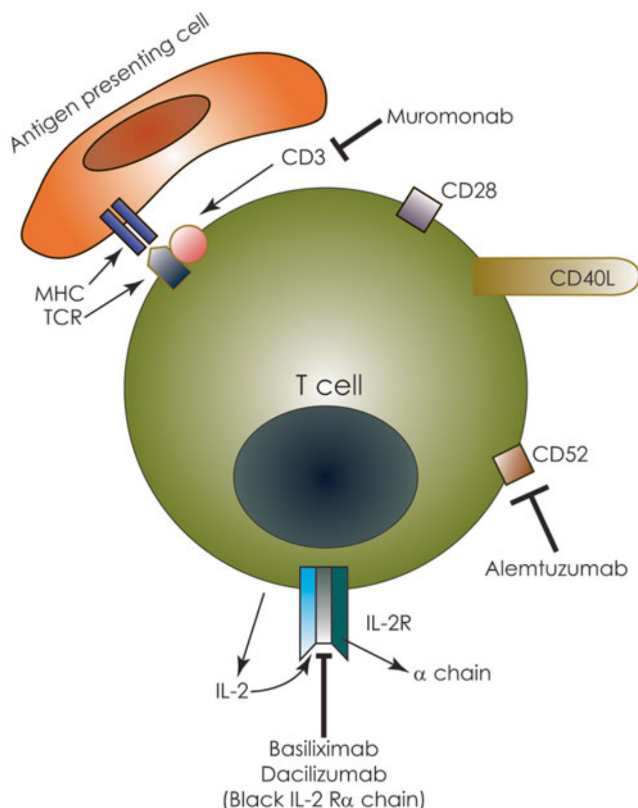


Fig. 5.4 This figure depicts the mechanism of action of monoclonal antibodies that block CD3 receptors (muromonab) IL-2 R α chain (daclizumab, basiliximab), and CD52 receptor (alemtuzumab) on T cells

A major side effect, a cytokine-release syndrome, is observed within 30–60 min after the administration of muromonab-CD3. The side effect could last for several days which is the result of increased production of several cytokines including IL-2, IL-6, TNF-α, and interferon-γ. TNF-α appears to be responsible for most of the discomfort, and the symptoms are more pronounced after the first administration of muromonab-CD3.

5.8.1.2 Daclizumab (Zenapax)

Daclizumab is an IL-2 receptor antagonist that binds to the Tac unit (CD-25) of the IL-2 receptor with high affinity, thus preventing the binding of IL-2 to its receptors. Daclizumab is a CDR-grafted (humanized) monoclonal antibody, which, is 90 % human IgG and 10 % murine antibody, and the murine part binds to CD-25 receptor.

Since Tac subunit of IL-2 receptors is expressed only on activated and not on resting T cells, its effects are restricted to activated T cells and may not alter the entire immune response (Fig. 5.4). In allograft rejection, IL-2-activated T cells play a critical role, and consequently inhibition of IL-2-dependent responses by daclizumab makes it an important drug for preventing the tissue rejection. It also interferes with the development of immune response to antigenic challenges and is suggested to inhibit expression of the IL-2 α and β chains and to cause a decrease in circulating lymphocytes.

Daclizumab is used for the prophylaxis of acute rejection in patients receiving kidney transplants. A dose of 1 mg/kg is sufficient to completely block all the IL-2 receptors. It is administered in five doses at a 2-week interval where its elimination half-life is about 20 days. A combination of several other immunosuppressive agents including cyclosporine (or tacrolimus, rapamycin), mycophenolate, mofetil, and corticosteroids can be used with daclizumab. When it is used in combination with tacrolimus, the doses of tacrolimus are reduced. After tissue transplantation, the addition of daclizumab to the standard immunosuppressive regimen produces reduction in tissue rejection up to 50 %. Daclizumab can cause hypersensitivity reactions, but it does not cause cytokine-release syndrome. There is also low incidence of opportunistic infections and lymphoproliferative disorders with daclizumab. When administered in combination with other immunosuppressive agents, it does not affect the side effects of other pharmacologic agents. However, increased incidence of wound infection and cellulitis has been reported when daclizumab is used in combination with other immunosuppressive agents (Fig. 5.4).

5.8.1.3 Basiliximab (Simulect)

Basiliximab, a glycoprotein produced by recombinant DNA technology, is a chimeric (human 60 %, murine 40 %) monoclonal antibody (IgG_{1K}) that binds to and blocks IL-2 receptor-α chain (CD-25), which is also known as Tac and is expressed only on activated T cells. It has higher affinity for IL-2 receptors than daclizumab. After binding to IL-2 receptor-α chain, basiliximab causes the inhibition of IL-2-induced T-cell activation, which is critical in the etiology and pathogenesis of the tissue rejection pathway (Fig. 5.4). As is the case for daclizumab, it also inhibits antigen-induced immune response.

Basiliximab is used for the prophylaxis of acute rejection for patients undergoing kidney transplantation where it is employed in combination with other standard immunosuppressive therapy regimens. After tissue transplantation, its addition to the standard immunosuppressive regimen results in inhibiting tissue rejection up to approximately 30 %. Both daclizumab and basiliximab have similar effects on the expression of IL-2 α and IL-β chains.

Basiliximab is contraindicated in patients with known hypersensitivity to this antibody. There is a slight increased risk of gastrointestinal discomfort as compared with placebo after treatment with basiliximab. However, there is no difference in the incidence of malignancies and infections between the patients receiving basiliximab and placebo. Furthermore, it does not increase the adverse effects resulting from the administration of a standard immunosuppressive regimen and other medications.

5.8.2 Psoriasis

Psoriasis is a disease of the immune system that involves T lymphocytes. The etiology and pathogenesis of psoriasis result from complex communications that cause activation of T lymphocytes and trafficking to the skin. Further reactivation causes inflammation and overproduction of the skin, resulting in lesions and plaques. In the psoriatic skin, there is an upregulation of intracellular adhesion molecule-1 (ICAM-1) on endothelium and keratinocytes. The immune cells move from the dermis to the epidermis and the inflammation is not caused by an outside antigen.

5.8.2.1 Ustekinumab (Stelara)

Ustekinumab is a recombinant human monoclonal antibody (IgG1α) directed against the p40 subunit of IL-23 and IL-12. Both IL-23 and IL-12 share p40 subunit. IL-23 is released by TH22 cells, dendritic cells, and macrophages and plays an important role in inflammation. In association with IL-6 and TGFβ, IL-23 induces naïve CD4+ T cells to differentiate into TH17 cells, which are distinct from TH1 and TH2 cells. TH17 cells produce IL-17, which is a pro-inflammatory cytokine that enhances T-cell priming and increases the production of IL-1, IL-6, TNFα, and several chemokines from keratinocytes. Another cytokine, IL-22, secreted by dendritic cells activates T cells causing the proliferation of keratinocytes, resulting in thick psoriatic plaques on the skin. Ustekinumab binds to p40 subunit and prevents the binding of IL-23 and IL-12 to their receptors, and as a consequence interferes with the induction of inflammatory response and the release of a number of other cytokines and chemokines. The mechanism of action of ustekinumab is depicted in Fig. 5.5. It is used to treat moderate to severe plaque psoriasis and psoriatic arthritis and also benefits patients who do not respond to Enbrel. For psoriatic arthritis, it is used alone or in combination with methotrexate. In patients weighing 220 lb or les, an initial dose of 45 mg is injected subcutaneously, followed by another dose in 4 weeks, and then the drug is administered once every 12 weeks. The adverse effects include allergic reactions, flu-like symptoms, mouth sores, rapid heartbeat, swelling of the body, pain or burning during urination, upper respiratory infection, increased risk of infection, increases risk of cancer, confusion, change in mental state, and brain swelling of posterior reversible encephalopathy syndrome. It is contraindicated in patients with a history of infections, cancer, immune system problems, or patients who have lived in areas where the prevalence of tuberculosis is high. Cyclosporine or warfarin may increase its side effects.

5.8.2.2 Secukinumab (Cosentyx)

Secukinumab is a human antibody which targets IL-17 and is used to treat psoriasis. It is specific for human IgG1k. This monoclonal antibody is also under investigation for ankylosing spondylitis, psoriatic arthritis, and autoimmune encephalomyelitis. The side effects of secukinumab include nasopharyngitis, diarrhea, upper

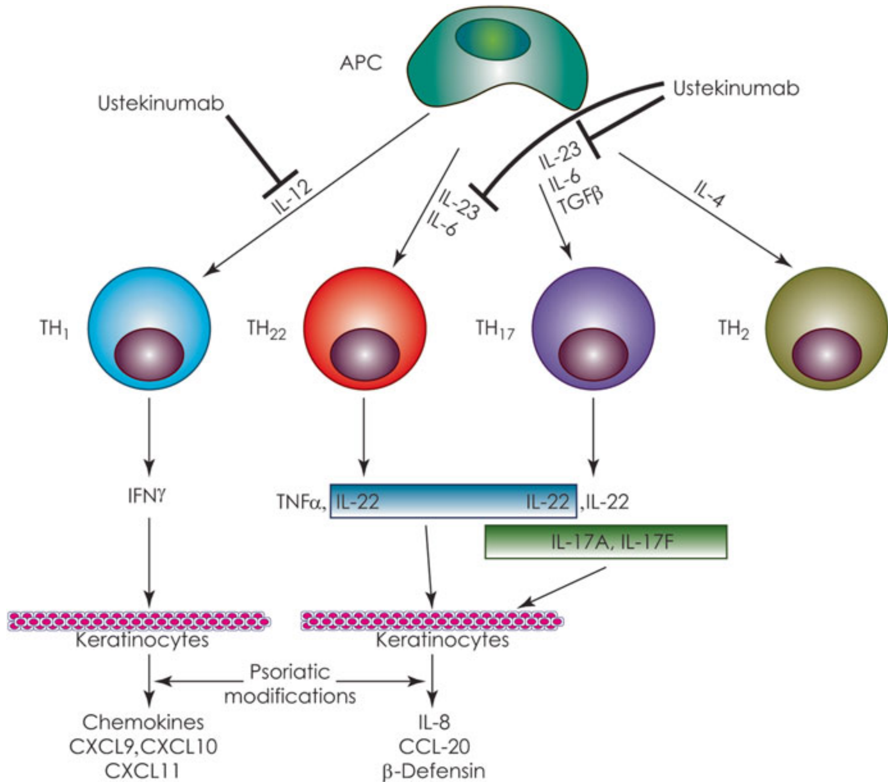


Fig. 5.5 Mechanism of action of ustekinumab: This monoclonal antibody used for the treatment of psoriasis is directed against the p40 subunit of IL-23 and IL-12. It prevents the binding of IL-23 and IL-12 to their receptors and inhibits the induction of the inflammatory pathways. IL-23 causes the differentiation of naïve T cells into TH17 cells that produce IL-17, a proinflammatory cytokine. Ustekinumab inhibits IL-12/23 pathway resulting in the inhibition of production of both TH17 and TH1 cells and their cytokines and chemokine. As a consequence, there is clearance of psoriasis

respiratory infection, rhinitis, oral herpes, pharyngitis, urticaria, muscle ache, shortness of breath, increased or painful urination, unexplained weight loss, and rhinorrhea. Some patients have exacerbations of Crohn's disease which could be serious.

5.8.3 Treatment of Autoimmune Diseases

5.8.3.1 Infliximab (Remicade)

Infliximab is a chimeric monoclonal antibody (IgG1K) that is produced by recombinant DNA technology and is directed against TNF-α. It is composed of human constant and mouse variable regions. Infliximab binds to the soluble and the membrane-bound form of TNF-α resulting in the neutralization of its biological activity. This is achieved via the inability of TNF-α to bind to its receptors in the

presence of infliximab. The prominent effects of TNF-α include enhanced production of IL-1 and IL-6, induction of adhesion molecules, and inflammatory cell traffic, as well as stimulation of acute phase response and tissue-degrading enzymes. Infliximab suppresses the expression of adhesion molecules, decreases IL-6 levels, inhibits acute phase response, and may alter other immune cells. It lyses the cells expressing transmembrane TNF-α receptors.

Infliximab is used to reduce signs, symptoms, and progression of autoimmune diseases. The drug is indicated for patients with moderate to severe active rheumatoid arthritis, where infliximab reduces infiltration of inflammatory cells into the inflamed areas of joints. Infliximab is also indicated in moderate to severe active Crohn's disease, psoriasis, ulcerative colitis, and ankylosing spondylitis. It is used to reduce signs and symptoms and to maintain clinical remission. The number of draining enterocutaneous and rectovaginal fistulas is reduced by infliximab. It helps maintain fistula closure in patients with fistulizing Crohn's disease.

Infliximab is administered in combination with methotrexate for rheumatoid arthritis. A dose of 3 mg/kg is administered via intravenous infusion and is repeated after 2 and 6 weeks followed by the maintenance dose every 8 weeks. The recommended dose for Crohn's disease is 5 mg/kg. The side effects associated with the administration of infliximab include acute infusion reactions (fever, chills, chest pain, hypotension, and rare anaphylaxis), increased risk of infection and production of autoantibodies (lupus-like syndrome), increased risk of malignancies, immunogenicity, cough, back pain, nausea, vomiting, and several leukocyte disorders.

5.8.3.2 Adalimumab (Humira)

Adalimumab, a human monoclonal antibody (IgG1) directed against TNF-α, is produced by phage display technology. It binds to TNF-α resulting in the inhibition of its ability to bind to its p55 and p75 cell surface receptors. Adalimumab modulates biological responses induced or regulated by TNF including expression of adhesion molecules, acute phase response, IL-1 and IL-6 levels, and metalloproteinases. It neutralizes the elevated levels of TNF found in the synovial fluid of patients with rheumatoid arthritis and psoriatic arthritis.

Adalimumab is used to reduce signs and symptoms and progression of rheumatoid arthritis, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, and ulcerative colitis. It could be administered alone or in combination with methotrexate. The recommended dose for adults is 40 mg subcutaneous injection, administered every other week. The most common side effect associated with the administration of adalimumab is injection site reaction. The most serious side effects resulting from treatment with this antibody include increased risk of infections, malignancies, and neurological disorders. Additional side effects are the production of autoantibodies, immunogenicity, and gastrointestinal disorders.

5.8.3.3 Golimumab (Simponi)

Golimumab is a human monoclonal antibody directed against TNF-α and is used as an immunosuppressive drug. It is administered once a month subcutaneously for the treatment of moderate to severe rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and ulcerative colitis (in adults). Sometimes it is used in combination with methotrexate. Patients taking abatacept, Kineret, or other TNF-α blockers should not be given golimumab. It may produce serious and sometimes fatal infections. The propensity to acquire infection increases, if the patient is already taking another immunosuppressive drug. It enhances the risk of developing lymphoma or melanoma. Some patients develop a rare fast-growing type of lymphoma which affects the liver, spleen, and bone marrow and can be fatal. Its risk is higher in teenage boys and young men. Cases of the development of lupus-like syndrome also occur.

The common side effects of golimumab include acute infection of the nose or throat, bacterial infection of blood or tissue, cellulitis, irritation of the injection site, and throat irritation. Other side effects include development or worsening of the heart failure, serious liver problems, low blood counts, and nervous system problems which are rare.

5.8.3.4 Tocilizumab (Actemra)

Tocilizumab is a humanized monoclonal antibody which is directed against IL-6 receptors. The drug binds to soluble as well as membrane-bound IL-6 receptors. It is used subcutaneously for the treatment of moderate to severe rheumatoid arthritis and systemic juvenile idiopathic arthritis, generally given in combination with methotrexate. However, it could be administered alone if the patient does not tolerate methotrexate. The treatment by tocilizumab is preferable, if other drugs such as disease-modifying antirheumatic drugs (DMARDs) and TNF-α blockers are not effective or tolerated by the patient. Its application is contraindicated during acute infection. The most common side effects of tocilizumab are upper respiratory tract infections, common cold, headache, high blood pressure, and elevated total cholesterol levels. It may elevate alanine transaminase in a few patients without symptoms. Infections, rashes, and allergic reactions have also been reported though rare in frequency.

5.8.3.5 Certolizumab (Cimzia)

Certolizumab pegol is a pegylated humanized Fab fragment of an anti-TNF-α monoclonal antibody. It neutralizes membrane-bound and soluble human TNF-α in a dose-dependent manner. Certolizumab pegol has a high affinity for TNF-α, but does not induce antibody-dependent cellular cytotoxicity, complement activation, or apoptosis in T cells or macrophages. It is not surprising, since it lacks Fc region of the antibody. Certolizumab is used for the treatment of rheumatoid arthritis and Crohn's disease. It may decrease body's ability to fight infection and increase the

risk of serious or life-threatening infections including severe bacterial, fungal, and/or viral infections. The adverse effects may include gastrointestinal discomfort, intestinal obstruction, diarrhea, upper respiratory infection, urinary tract infection, and arthralgia. It may also cause heart failure or worsening of the symptoms, allergic reaction, demyelinating disease, and lupus-like syndrome.

5.8.3.6 Vedolizumab (Entyvio)

Vedolizumab is a recombinant humanized IgG1 kappa isotype monoclonal antibody targeted against integrin α4β7 (LPM-1, lymphocyte Peyer's patch adhesion molecule). It blocks the binding of integrin α4β7 to mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The blood vessels of the intestinal tract preferentially express MAdCAM-1. Inhibition of integrin α4β7 produces anti-inflammatory response specific for the gut, via suppression of adhesion and migration of leukocytes into gastrointestinal tract. It is used to treat ulcerative colitis and Crohn's disease. The adverse effects include allergic reactions, increase risk of infection, progressive multifocal leukoencephalopathy, liver damage, flu-like symptoms, gastrointestinal disorders, tiredness, joint pain, back pain, pain in the extremities, common cold, and infection of the nose and throat. Vedolizumab should not be given to the patients who have infection, a history of recurrent infections, liver problems, and previous contact with a tuberculosis patient. It may also increase the risk of gastrointestinal infections due to its specificity for the gut.

5.8.3.7 Belimumab (Benlysta)

Belimumab is a human monoclonal antibody that inhibits B-cell activating factor. B-cell activating factor (BAFF) is also referred to as B-lymphocyte stimulator (Fig. 5.6). The growth factor is responsible for the development and survival of B cells and is synthesized by a variety of cell types, including macrophage, monocytes, bone marrow stromal cells, and astrocytes. BAFF binds to three distinct receptors, BAFF receptor (BAFF-R), B-cell maturation antigen (BCMA), and transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), on B cells. As described previously, B cells develop in the bone marrow and continue to mature in secondary lymphoid organs and in the gut. B cells secrete antibodies, and in case of autoimmune diseases, some release autoimmune antibodies. However, autoimmune B cells are normally destroyed by apoptosis. BAFF is overexpressed in SLE, and it is assumed that in SLE autoantibody-secreting B cells continue to proliferate and are protected by the growth factor. As a result, this antibody is used to target the growth factor. Belimumab reduces the number of circulating B cells, but anti-CD20 monoclonal antibodies reduce the number even more.

It is possible that belimumab binds primarily to the circulating soluble BAFF, therefore not inducing antibody-dependent cellular cytotoxicity that could be expected from this IgG1-type antibody. Belimumab is administered to treat systemic

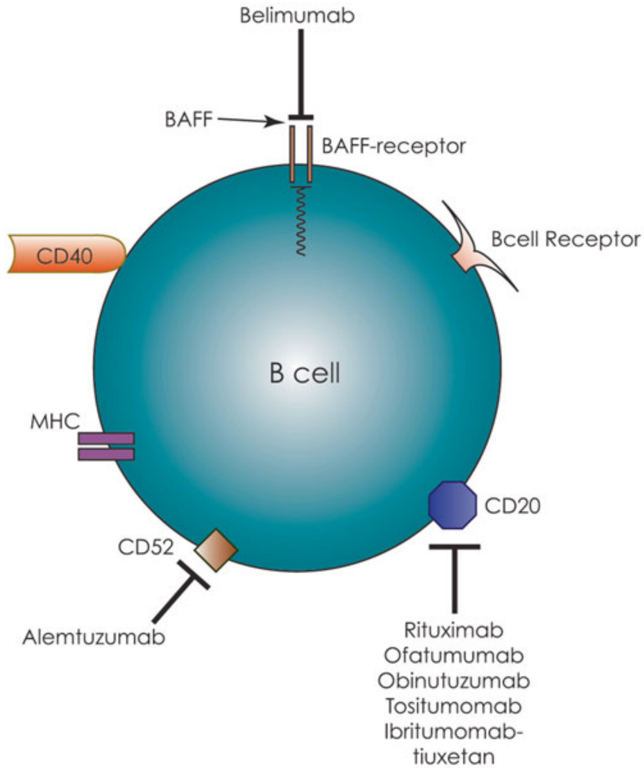


Fig. 5.6 Monoclonal antibodies directed against CD20 antigen receptors and B-cell activating factor: These antibodies are used to treat a number of B-cell malignancies, and the latter is used to treat systemic lupus erythematosus

lupus erythematosus. Administration of antihistamine prior to its infusion is suggested. It is not yet approved in SLE patients with severe kidney and brain damage. There are concerns that it is only marginally effective. In clinical trials, black/African-American patients did not benefit from the drug, while in other patients, it reduced disease activity and severe flare ups. Due to its immunosuppressive activity, there is an increased risk of infection. The side effects of belimumab include nausea, diarrhea, fever, hypersensitivity, and infusion-site reactions.

5.8.3.8 Natalizumab (Tysabri)

Natalizumab is a humanized monoclonal antibody which is specific for α-4 integrin. It is used for the treatment of multiple sclerosis and Crohn's disease. The lesions in multiple sclerosis are caused by T lymphocytes passing through the blood–brain barrier via their interaction with the adhesion molecules on endothelial cells (high endothelial venules). To facilitate this cellular traffic, α-4 integrins on T lymphocytes interact with VCAM-1 on endothelial cells, since this effect is observed in

endothelial cells expressing VCAM-1. Involvement of parenchymal cells expressing osteopontin gene has also been reported. The administration of natalizumab decreases the migration of leukocytes into the brain's parenchyma in animal models. This effect is accompanied by a decrease in lesions; however, the significance of these observations is uncertain for the treatment of disease in human. Natalizumab interferes with the cellular traffic through the cellular lining of the blood–brain barrier and intestines, and therefore produces its anti-inflammatory effects. There is an increase in the expression of CD34 baring cells in patients treated with natalizumab. CD34 is a cell surface glycoprotein which plays a role in cell–cell interaction. It is also an adhesion molecule and is required by T cells to enter the lymph nodes. In patients with multiple sclerosis, natalizumab prevents relapses, cognitive decline, vision loss, and improves the quality of life.

Crohn's disease is characterized by chronic bowel inflammation which seems to result from the interaction of the α4β7 integrin and MADCAM1 (addressin) and subsequent inflammatory cell migration. The α4β7 integrins are expressed on leukocytes, and MADCAM1 receptors are expressed on endothelial cells. Specifically, MADCAM1 receptors are primarily expressed on high endothelial venules in the small intestine and are crucial for the migration of T lymphocytes into lymphatic tissues in Peyer's patches. There is an increased expression of MADCAM1 at the active site of the bowel inflammation in patients with Crohn's disease. Natalizumab may interfere with the interaction of α4β7 integrin and MADCAM1 at the site of inflammation. In patients with Crohn's disease, natalizumab increases rates of remission and delays relapses.

The most adverse side effect of the drug is progressive multifocal leukoencephalopathy (PML), which is an opportunistic infection caused by John Cunningham (JC) virus. The risk for the infection increases when the drug is used in combination with another immunosuppressive or immunomodulatory agent. Other adverse effects include injury to the liver, nausea, headache, cold, fatigue, allergic reaction, and exacerbation of Crohn's disease in a few patients with the disease. The anaphylactic reaction to natalizumab is rare. Hepatotoxicity includes increased levels of bilirubin and liver enzymes. There have been reports of its links to melanoma; however, this association has not been clearly established. It is contraindicated in patients with immunosuppressive state and/or a history of progressive multifocal leukoencephalopathy.

5.8.3.9 Canakinumab (Ilaris)

Canakinumab is a human monoclonal antibody which is specific for IL-1β belonging to IgG1/κ isotype subclass and is not cross-reactive to other members of IL-1 family. It is used for the treatment of cryopyrin-associated periodic syndromes (CAPS). These syndromes are a group of auto-inflammatory diseases that include familial cold auto-inflammatory syndrome, neonatal-onset multisystem inflammatory disease, systemic juvenile idiopathic arthritis, and Muckle–Wells syndrome. The dose for CAPS patients with body weight greater than 40 kg is 150 mg, and for

patients between 15 and 40 kg, the dose is 2 mg/kg. For children weighing between 15 and 40 kg who have not responded well, the dose can be increased to 3 mg/kg. It is administered subcutaneously every 8 weeks. For systemic juvenile idiopathic arthritis (SJIA) patients with a body weight more than or equal to 7.5 kg, the dose is 4 mg/kg (with a maximum of 300 mg). It is administered subcutaneously every 4 weeks. The side effects may include allergic reactions, difficulty in breathing, hives, swelling of face, lip, tongue, or throat. Other adverse reactions associated with canakinumab are flu-like symptoms, sore throat, chills, fever, nausea, vomiting, loss of appetite, mouth sores, gum bleeding, cough, chest pain, dizziness, and shortness of breath.

5.8.4 Treatment of Thrombosis

5.8.4.1 Abciximab (ReoPro)

Abciximab is a Fab fragment (7E3) of a chimeric monoclonal antibody that is directed against members of integrin GPIIb/IIIa receptor family of adhesion molecules. These receptors are located on platelets where they are involved in platelet aggregation. Abciximab inhibits platelet aggregation by blocking GPIIb/IIIa receptors, thus preventing the binding of fibrinogen, von Willebrand factor, and other molecules promoting adhesion to the receptors on platelets. It increases bleeding and activated clotting times and reduces the response of platelets to adenosine diphosphate. The Fab fragment of abciximab does not directly interact with the arginine–glycine–aspartic acid (RGD) binding site of GPIIb/IIIa but appears to be involved in steric hindrance, resulting in interference for other larger molecules.

The antibody fragment also binds to vitronectin receptors (aV integrin) that are present on a number of tissues including vessel wall endothelial cells, smooth muscle cells, and platelets. These receptors are involved in the proliferative properties of endothelial and smooth muscle cells and as well as the procoagulant properties of the platelets.

Abciximab is indicated in patients with heart disease caused by poor blood flow in the arteries of the heart (ischemic cardiac complications). It is also used in patients with unstable angina not responding to conventional treatment and for percutaneous coronary intervention. It can reduce the incidence of abrupt closure and restenosis resulting from percutaneous transluminal coronary angioplasty. Abciximab may also be used in unstable angina and acute therapy of myocardial infarctions.

An initial bolus dose of 0.25 mg/kg of abciximab is administered intravenously followed by continuous infusion of 5 or 10 mg/min for 12–96 h. Following an IV administration, its initial half-life is of several minutes followed by a second-phase half-life of about 30 min, which is attributed to rapid binding to its receptors on the platelets. Within 10 min after administration, abciximab exhibits its inhibitory effects on platelet aggregation after a single bolus injection and remains in circulation for several days bound to platelets. The side effects of abciximab include irritation at the

injection site, nausea, vomiting, dizziness, and bleeding. Occasional breathing trouble, rapid or abnormal heart beat, chest pain, and swelling of the feet or ankles may occur after its administration. Human anti-chimeric antibodies (HACA) may be produced in response to abciximab.

5.8.5 *Treatment of Cancer*

5.8.5.1 Rituximab (Rituxan)

Rituximab is a chimeric (murine/human) monoclonal antibody (IgG_1K) specific for cells possessing a CD20 antigen, which is a human B-lymphocyte-restricted differentiation antigen also termed as Bp35 or B1. It is distributed on pre-B cells and with a higher density on mature B cells but is not present on hematopoietic stem cells, pro B cells, and plasma cells (Table 5.1). CD20 is a transmembrane phosphoprotein, which is also expressed on more than 90 % of B-cell non-Hodgkin's lymphomas. It does not shed from its cell surface membrane-bound form and also does not internalize after binding to its antibody. CD20 antigen is not expressed on most tissues and regulates cell cycle initiation and differentiation. Rituximab contains human constant region and murine light and heavy chain variable region, and its Fab domain binds to CD20 antigen on B cells and Fc domain recruits immune effector cells and mediators for B-cell lysis. The lysis can be achieved by antibody-dependent cell-mediated cytotoxicity and apoptosis (Fig. 5.6).

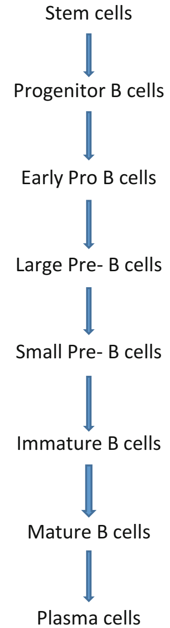
Rituximab is used for the treatment of relapsed or refractory, low-grade or follicular B-cell lymphoma ($CD20^+$), and non-Hodgkin's lymphoma. In combination with CHOP or other standard chemotherapeutic regimens, rituximab is the treatment for diffuse large B-cell, $CD20^+$, non-Hodgkin's lymphoma. The suggested dose is 375 mg/m^2 intravenous infusion, and generally four to eight doses are administered once a week. Fifty percent of patients have responded to the treatment with full or partial remission.

Most side effects are felt after the first treatment with rituximab and attention should be given to the rate of infusion. The most common immediate side effects of rituximab are fever, chills, and respiratory symptoms, but these effects are much milder than the traditional chemotherapy. Other infusion reactions include nausea, angioedema, headache, hypotension, pruritus, urticaria, rash, and vomiting. The adverse effects decrease with each subsequent administration of the drug. Other side effects associated with rituximab include B-cell depletion, cytopenia, immunogenicity, and multiple pulmonary events.

5.8.5.2 Ofatumumab (Arzerra)

Ofatumumab is a human monoclonal antibody specific for CD20 protein (Fig. 5.6). Its epitope is distinct from rituximab. It inhibits early stage B-cell activation. When compared with rituximab, ofatumumab's binding to CD20 is tighter and exhibits a

Table 5.1 Developmental stages of B cells



slower off-rate. It is cytotoxic to cells expressing CD20. This mechanism is mediated via complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC). It is used to treat chronic lymphocytic leukemia which is refractory to fludarabine and alemtuzumab (Campath). The most common side effects of ofatumumab include upper respiratory infection, lower respiratory infection, rash, neutropenia, and anemia.

5.8.5.3 Obinutuzumab (Gazyva)

Obinutuzumab is a humanized monoclonal antibody specific for CD20 receptor (Fig. 5.6). It is cytotoxic to cells expressing CD20 and kills B cells. The antibody acts as an immunomodulator. Obinutuzumab is used to treat chronic lymphocytic leukemia in combination with chlorambucil in patients who have not been treated previously with chemotherapeutic agents. Each dose of the drug is 1,000 mg with the exception of initial infusions in cycle I. On day 1 and 2 of the cycle I, doses of 100 mg and 900 mg are infused, respectively. Several premedications including glucocorticoids, acetaminophen, and antihistamine are given to the patient. The adverse effects may include hepatitis B virus reactivation, progressive multifocal leukoencephalopathy, infusion site reactions, neutropenia, thrombocytopenia, increased risk of infection, and immunogenicity in rare cases.

5.8.5.4 Trastuzumab (Herceptin)

Trastuzumab is a CDR-grafted (humanized) monoclonal antibody (IgG_1K) produced by recombinant DNA technology. It binds to human epidermal growth factor type 2-receptor (HER-2). Trastuzumab is composed of two antigen-specific sites which bind to extracellular domain of the HER-2 receptors resulting in inhibiting the induction of its intracellular tyrosine kinase. The rest of the antibody is human IgG, which has a conserved Fc portion. The proposed mechanisms by which trastuzumab may inhibit signal transduction involve antagonism of HER-2 receptor dimerization, activation of immune response, suppressing the shedding of the extracellular domain, and/or enhanced endocytic breakdown of the receptor (Fig. 5.7). HER-1, HER-2, HER-3, and HER-4 (also called epidermal growth factor receptors Erb B-1, Erb B-2, Erb B-3, and Erb B-4, respectively) are transmembrane tyrosine kinase receptors, which are partially homologous. These receptors regulate a variety of biological functions including cell growth, differentiation, migration, adhesion, survival, and their responses. The cell proliferation via HER-2 signaling is mediated through RAS-MAPK pathway, and cell death is inhibited via the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (mTOR) pathway. In 20–30 % of women with invasive breast carcinomas, there is a genetic alteration, which results in the overexpression of the HER-2 gene. This causes an increased amount of the growth factor receptor protein on the tumor cell surface and also results in elevated levels of circulating shed fragments of its extracellular domain. The HER-2 overexpression is associated with more aggressive metastatic breast cancer. Trastuzumab inhibits the proliferation of tumor cells overexpressing HER-2. It causes lysis via antibody-dependent cell-mediated cytotoxicity.

Trastuzumab is indicated as a single agent for the treatment of patients whose breast cancer has an overexpression of the HER-2 protein and has been previously treated by standard chemotherapeutic regimen. Trastuzumab is administered in combination with paclitaxel in patients who have not previously treated with chemotherapy and their tumors overexpress HER-2. The initial dose for trastuzumab is 4 mg/kg given as a 90 min infusion, and the weekly maintenance dose is 2 mg/kg administered as a 30 min infusion. Its half-life is 1.7 and 12 days when a dose of 10 and 500 mg is administered, and the volume of distribution is 44 mL/kg, which is serum volume on average.

Trastuzumab causes the development of ventricular dysfunction and congestive heart failure. The patients treated with trastuzumab have exhibited symptoms of cardiac dysfunction including dyspnea, increased cough, peripheral edema, and reduced ejection fraction. Congestive heart failure could also be severe. Left ventricular function must be evaluated in all patients before and during trastuzumab therapy. Patients receiving trastuzumab in combination with anthracycline and cyclophosphamide exhibit more severe cardiomyopathy. Other side effects associated with trastuzumab when administered in combination with chemotherapeutic agents include anemia and leukopenia and increased risk of upper respiratory infections, but when given alone, it does not produce frequent hematological toxicity. Trastuzumab may also cause severe hypersensitivity reactions including anaphylaxis and pulmonary events. Infusion-associated symptoms including chills and fevers have been reported in about 40 % of patients during the first infusion of trastuzumab.

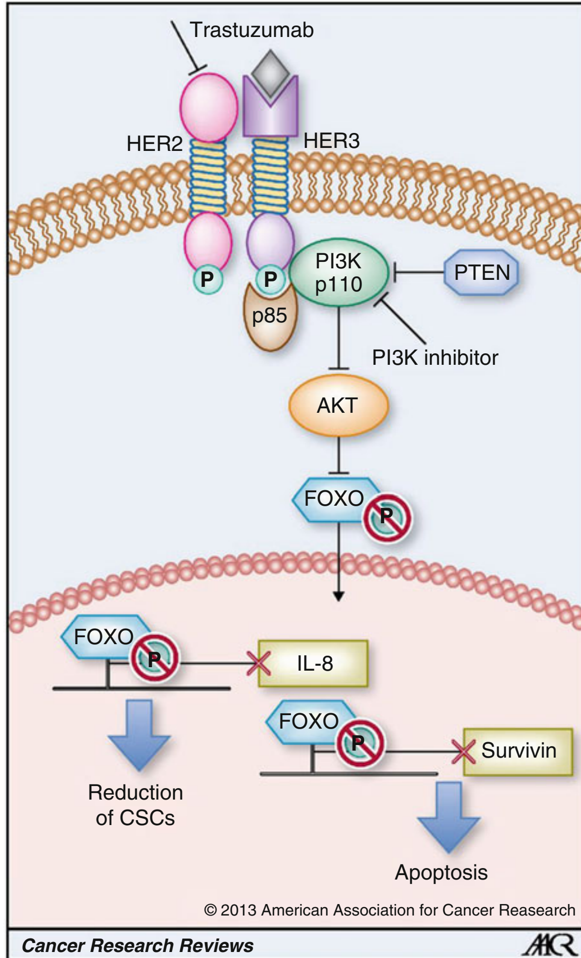


Fig. 5.7 Mechanism of action of trastuzumab: Trastuzumab binds to HER2 and interferes with the effect of human epidermal growth factor (Reproduced with permission, Source: Rexer BN, Arteaga CL (2013) Optimal targeting of HER2-PI3K signaling in breast cancer: mechanistic insights and clinical implications. *Cancer Res* 73:3187)

5.8.5.5 Pertuzumab (Perjeta)

Pertuzumab is a humanized monoclonal antibody that is a HER dimerization inhibitor. It binds to HER2 (proto-oncogene Neu, or ERBB2) and inhibits the dimerization of HER2 to other HER receptors. Pertuzumab is used in patients for the treatment of HER2-positive metastatic cancer in combination with trastuzumab and docetaxel, who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease. The side effects of pertuzumab include cardiomyopathy, embryo-fetal toxicity, hypersensitivity reactions/anaphylaxis, fever, and chills. The most

common side effects of the combination therapy are nausea, fatigue, diarrhea, alopecia, neutropenia, peripheral neuropathy, and rash.

5.8.5.6 Alemtuzumab (Campath)

Alemtuzumab is a CDR-grafted (humanized) monoclonal antibody (IgG1kappa) produced by recombinant DNA technology. It binds to CD52, which is a 21–28 Kd cell surface glycoprotein. This receptor is present on the cell surface of normal and malignant T and B cells, NK cells, and monocytes/macrophages. It mediates its effects via antibody-dependent lysis of target cells after binding to CD52 receptors (Figs. 5.4 and 5.6).

Alemtuzumab is prescribed for the treatment of B-cell chronic lymphocytic leukemia. It has also been used for refractory celiac disease. Alemtuzumab is administered daily by a 2 h IV infusion with an initial dose of 3 mg and the dose is then increased to 10 mg in patients who do not encounter serious side effects. If tolerating a dose of 10 mg/day is not a problem, a maintenance dose of 30 mg/day is administered on alternate days (three times/week), which is generally initiated in most patients in 3–7 days. The average half-life of the drug over the dosing interval is about 12 days, and a steady-state level is achieved in about 6 weeks after initiation of 30 mg dose. The side effects associated with alemtuzumab include immunosuppression/opportunistic infections, myelosuppression, and infusion-related adverse events resulting in fever, fatigue, pain, anorexia, and edema. It is not administered to patients with active systemic infection, AIDS, or hypersensitivity to alemtuzumab.

5.8.5.7 Cetuximab (Erbitux)

Cetuximab is a chimeric monoclonal antibody produced by hybridoma technology and is composed of murine Fv region and human IgG_1 (heavy and Kappa light chain constant regions) molecule. The murine region is specific for human epidermal growth factor receptor (EGFR, HER1, c-ERB-1). Epidermal growth factor receptors belong to the tyrosine kinase type 1 receptor subfamily, which also includes HER2, HER3, and HER4. Epidermal growth factor receptor, a transmembrane glycoprotein, is distributed on epithelial tissue as well as on the tumors of the colon, rectum, head, and neck.

Cetuximab is an antagonist of the EGF and its other ligands including transforming growth factor-α. It causes the inhibition of EGF-mediated signal transduction events including the phosphorylation and activation of various kinases associated with EGFR. The inhibition of the binding of EGF to its receptors suppresses cell growth and decreases the production of matrix metalloproteinase and vascular endothelial growth factor. Cetuximab after binding to EGFR induces apoptosis.

Cetuximab is indicated for the treatment of head, neck, and colorectal cancers, and in combination with radiation therapy, it is used for the treatment of squamous

cell carcinoma (locally or regionally advanced) of the head and neck. However, in patients with recurrent or metastatic squamous cell carcinoma of the head and neck whose response to the previous platinum-based therapy has not been positive, it is not given in combination with radiation therapy and is solely administered. Generally, a dose of 400 mg/m^2 is initially administered in combination with radiation therapy followed by a maintenance dose of 250 mg/m^2 and is administered 1 h before the radiation therapy. Similar doses are used when it is employed as a single agent.

Cetuximab is used for the treatment of metastatic colorectal carcinoma which expresses EGFR, in combination with irinotecan, provided that these patients are refractory to irinotecan-based standard chemotherapeutic regimen. For colon cancer, it is only administered to patients who do not have KRAS (GTPase KRAS also known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutation. KRAS is an on and off switch, which when turned on is responsible for cellular growth. Cetuximab has no effect on colon cancer which is due to KRAS mutation. The mutated KRAS gene continuously sends a signal downstream without responding to the EGFR. Cetuximab is only given after the KRAS gene is tested for the presence of the wild-type gene. In patients who could not tolerate irinotecan-based chemotherapeutic regimen, it is used as a single treatment for EGFR-expressing metastatic colorectal cancer. The doses are similar to the doses used for the squamous cell carcinoma.

It is also used in metastatic non-small cell lung cancer and head and neck cancer. Since this antibody is IgG1 isotype, it may activate the complement pathway and cause antibody-dependent cellular toxicity. One of the most serious side effects is acne-like rash.

Whether administered in combination or as a single therapy for the cancers of the head, neck, and colon, cetuximab exhibits similar pharmacokinetic characteristics. After a 2 h infusion of 400 mg/m^2, the half-life is 97 h, ranging from 41 to 213 h, and after initial and subsequent maintenance doses, the half-life is about 112 h, ranging from 63 to 230 h. The adverse effects associated with cetuximab include immunogenicity, electrolyte depletion (hypomagnesemia), and infusion reactions. Infusion reactions involve airway obstruction, urticaria, and hypotension.

5.8.5.8 Panitumumab (Vectibix)

Panitumumab is a fully human monoclonal antibody that is used to treat EGFR-expressing metastatic colorectal cancer. It does not work in patients who have KRAS or NRAS mutations. Panitumumab binds to the extracellular domain of epidermal growth factor receptor and prevents the activation of its signal-dependent cascade. These receptors belong to the family of tyrosine kinases and utilize MAP kinases for signal transduction. Since it is IgG2 isotype, it may not activate the complement-mediated pathway and antibody-dependent cellular cytotoxicity. Its pharmacokinetics shows the target-mediated disposition behavior. The drug causes skin rash, nausea, diarrhea, fatigue, and decreased magnesium levels.

5.8.5.9 Bevacizumab (Avastin)

Bevacizumab is a recombinant CDR-grafted (humanized) monoclonal antibody (IgG1) that is directed against human vascular endothelial growth factor (VEGF). Its antibody-binding region is murine-specific for VEGF. VEGF promotes the proliferation of endothelial cells and the formation of new blood vessels. The receptors (Flt-1 and KDR) for VEGF are distributed on the endothelial cells. Bevacizumab binds to VEGF and inhibits its binding to VEGF receptors on endothelial cells.

In combination with a standard chemotherapeutic regimen (5-fluouracil based), bevacizumab is used for the treatment of patients with malignant colon or rectal cancer, where it is indicated for the first- or second-line therapy. Bevacizumab in combination with bolus IFL is administered at a dose of 5 mg/kg and 10 mg/kg in combination with FOLFOX4. It is administered as intravenous infusion. Its average half-life is approximately 20 days (ranging from 11 to 50 days) and the steady state is reached in 100 days.

The adverse effects associated with bevacizumab include gastrointestinal perforation, hemorrhage, hypertension, complications in wound healing, nephritic syndrome, congestive heart failure, and arterial thromboembolic events. The patients receiving bevacizumab commonly experience pain, asthenia, headache, abdominal pain, nausea, vomiting, anorexia, upper respiratory infection, and exfoliative dermatitis.

5.8.5.10 Ipilimumab (Yervoy)

Ipilimumab is a human antibody used for the treatment of malignant melanoma. It binds to CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4). CTLA-4 is expressed on the surface of helper T cells and regulatory T cells and transmits inhibitory signals to T cells. The target for CTLA-4 is CD80 and CD86 (B7) on antigen-presenting cells; as a result, it downregulates disease-fighting T-cell responses. Ipilimumab blocks the function of CTLA-4, resulting in a sustained immune response that attacks the cancer cells. Cytolytic T lymphocytes ($CD8^+$) play a role in recognizing and destroying cancer cells, but an inhibitory mechanism interrupts this destruction. Ipilimumab blocks this inhibitory mechanism and allows cytolytic T cells ($CD8^+$) to continue to destroy cancer cells. The drug causes an increase in the percentage of activated HLA-DR^+ T cells and a mean decrease in the percentage of $CCR7^+$ $CD45RA^+$ T cells. Ipilimumab is used for the treatment of malignant melanoma and is being evaluated to treat small cell lung cancer (SCLC), non-small cell lung carcinoma (NSCLC), bladder cancer, and metastatic hormone-refractory prostate cancer. It is administered in a total of four doses, intravenously, 3 mg/kg over 90 min every 3 weeks. The combination of ipilimumab and leflunomide may result in hepatotoxicity. Less severe adverse drug–drug interactions are associated with approximately 100 other drugs.

The most adverse effect associated with ipilimumab treatment is severe and potentially fatal immunologic adverse reactions, as a result of T-cell activation and

proliferation. These adverse reactions include inflammation of the intestine that may tear its walls, inflammation of the liver resulting in severe liver damage, inflammation of the nerves that potentially may cause paralysis, inflammation of the skin, inflammation of the eyes, and inflammation of some glands. Less severe adverse effects involve gastrointestinal tract including bloating, constipation or diarrhea, and stomach pain. Other side effects are fever, breathing and/or urinating problems, easy bruising or bleeding, blistering or peeling skin, sores in the mouth, skin rash that may or may not be itchy, unusual weakness of the limbs or face, and headaches. Numbness or tingling in the hands or feet, feeling cold all the time, decreased sex drive, irritability, forgetfulness, weight gain, dizziness, changes in mood or behavior, fainting, and vision problems may also occur.

5.8.5.11 Pembrolizumab (Keytruda)

Pembrolizumab is a humanized monoclonal antibody that is a class of drugs called PD-1 inhibitors. It is approved for the treatment of metastatic melanoma and is specific for programmed cell death 1 (PD-1) receptor. Programmed cell death protein is a cell surface protein molecule which is encoded by PCD1 gene and is a member of the extended CD28/CTLA-4 family of the regulators of T cells. This gene is responsible for coding a protein on the surface of cells that belongs to immunoglobulin superfamily. It is also called CD279 and is present on the cell surface of T and Pro-B cells. CD279 is involved in the differentiation of these lymphocytes. The programmed cell death 1 (PD-1) has two ligands, PCDL1 and PCDL2, which are members of B7 family, and negatively regulate immune responses. PD-1 and its ligands, PD-L1 and PD-L2, suppress the immune response by inhibiting the induction of T cells (Fig. 5.8). GM-CSF and LPS upregulates PD-L1 on macrophages and dendritic cells, whereas, TCR and B-cell receptor signaling results in induction of its expression on T cells and B cells. Overall, PD-1 negatively regulates T-cell responses. This results in self-tolerance and protects from autoimmune diseases. The suppressive effects of PD-1 are mediated by apoptosis of antigen-specific T cells in lymph nodes and inhibition of apoptosis of Treg cells.

Pembrolizumab is used after treatment with ipilimumab. It is indicated for patients whose tumors express BRAFV600 and have already been treated with both ipilimumab and a BRAF inhibitor (vemurafenib or dabrafenib). BRAF is a human gene that is responsible for the translation of protein B-Raf and undergoes mutation in some forms of cancer. These patients have few therapeutic options. Pembrolizumab is usually administered at a dose of 2 mg/kg or higher dose of 10 mg/kg. The adverse effects of pembrolizumab include nausea, decreased appetite, constipation, diarrhea, fatigue, rash, pruritus, cough, and arthralgia. The drug can also cause severe immune-mediated adverse effects which may involve healthy organs including the liver, lung, colon, and hormone-producing glands.

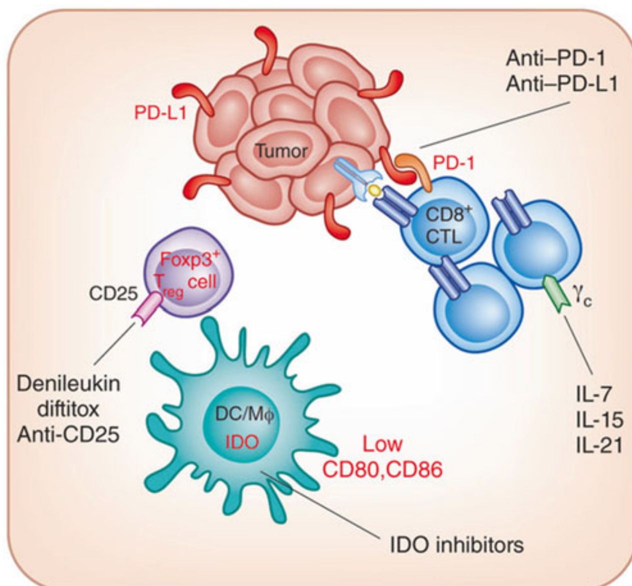


Fig. 5.8 The role of PD1 and PDl-1 in cancer: PD-1 functions as "immune checkpoint" and inhibits the immune response after binding to two ligands PDL-1 and PDL-2. The highest expression of PD-L1 is seen in T cell-infiltrated tumors. The tumors expressing PDL-1 have poor prognosis. The antagonism of PD-1 and PDL-1 by monoclonal antibodies which block their interaction results in the proliferation of T cells which could attack the tumor cells (Reproduced with permission. Source: Gajewski TF, Schreiber H, Fu Y-X (2013) Innate and adaptive immune cells in the microenvironment. *Nature Biol* 14:1014–1022)

5.8.5.12 Nivolumab (Opdivo)

Nivolumab is a human IgG4 monoclonal antibody directed against programmed cell death (PD-1) receptor on induced T cells. It blocks the activation of PD-1 receptors though their ligand. PD-1 then allows the immune response to be produced in the close vicinity of the tumor. The drug is used for the treatment of unresectable or metastatic melanoma in patients who no longer respond to other drugs and advanced (metastatic) squamous non-small cell lung cancer. For lung cancer, it is used for patients who have previously been treated with platinum-based chemotherapy. The manipulation of T-cell induction via inhibition of the PD-1 system produces anticancer response in non-small cell lung cancer. The side effects of nivolumab include fatigue, shortness of breath, a lack of appetite, nausea, constipation, and musculoskeletal pain. More serious adverse reactions involve immune-mediated effects on the lung, liver, kidneys, colon, and endocrine system.

5.8.5.13 Trastuzumab Emtansine (Kadcyla)

Trastuzumab emtansine is a conjugated monoclonal antibody which is composed of trastuzumab (Herceptin) linked to the cytotoxic agent mertansine. Mertansine enters tumor cells, binds to tubulin, and kills them. The conjugate is only delivered to the cells expressing HER2, which is overexpressed in tumor cells. The drug is used specifically in patients who are afflicted with HER2-positive metastatic breast cancer and have been previously treated with Herceptin and paclitaxel or docetaxel. It is also indicated for patients who have already been treated for metastatic breast cancer or have redeveloped tumor within 6 months of adjuvant therapy. The most common adverse effects of the drug include nausea, fatigue, headache, constipation, musculoskeletal pain, increased liver enzyme levels, and thrombocytopenia. Rare cases of heart, liver, and lung damage have also been reported.

5.8.5.14 Gemtuzumab-Ozogamicin (Mylotarg)

Gemtuzumab-ozogamicin is a conjugated CDR-grafted (humanized) monoclonal antibody (IgG4Kappa) that is conjugated to calicheamicin, a cytotoxic chemotherapeutic agent. The antibody is directed against CD33 antigen, which is expressed on the cell surface of leukemic blast cells and normal cells of myelomonocytic lineage, but is not found on normal hematopoietic stem cells. Gemtuzumab-ozogamicin is indicated for the treatment of CD33-positive acute myeloid leukemia. More than 80 % of patients with acute myeloid leukemia (AML) express CD33 antigen on the cell surface of the leukemic blast cells. Gemtuzumab binds to CD33 antigen via its antibody fragment, resulting in a complex that is subsequently internalized. The internalization causes the release of calicheamicin derivative inside the lysosomes where the cytotoxic agents then bind to DNA in the minor groove and cause double-strand breaks in the DNA and cell death.

Gemtuzumab-ozogamicin is administered as a dose of 9 mg/m^2 and is infused over a 2 h period; prior to its infusion, the reduction of leukocytes in patients to below 30,000/mL is recommended. A second dose is administered after 14 days interval. The elimination half-life of total calicheamicin is about 41 h after the first dose, 64 h after the second dose, and in its unconjugated form it is 143 h. The most common side effects associated with gemtuzumab-ozogamicin are fever and chills. Other adverse effects resulting from the administration of gemtuzumab-ozogamicin include myelosuppression, thrombocytopenia, neutropenia, increased risk of infection, abdominal pain, vomiting, and headache.

5.8.5.15 Ibritumomab (Zevalin)

Ibritumomab, a murine monoclonal antibody (IgG1Kappa), is first conjugated to a linker–chelator tiuxetan. Tiuxetan is responsible for a high affinity and conformationally restricted chelation to one of the two radiation sources: Indium-111 or

Yttrium-90. The antibody is directed against CD20 antigen that is not shed from the cell surface and is not internalized after binding to the antibody (Fig. 5.6). The antibody for treatment is supplied in two vials, one containing antibody conjugated to Indium-111 and the other to Yttrium-90. After binding to cells expressing CD20 antigen, ibritumomab delivers radiation which enhances the killing effect of the antibody.

Ibritumomab along with radioisotopes, which constitutes its therapeutic regimen, is indicated for the treatment of relapsed or refractory low-grade follicular, or transformed B-cell non-Hodgkin's lymphoma. It is also used in patients with rituximab refractory follicular non-Hodgkin's lymphoma. Ibritumomab therapy involves two steps. The patient initially receives a single infusion of rituximab (250 mg/m^2), which is administered initially to clear the majority of B cells and to limit the toxicity from radiation. The immunoconjugate is linked to Indium 111 for the first transfusion, 5.0 mCi of which is administered intravenously. Seven to 9 days after the first infusion, a second infusion of rituximab (250 mg/m^2) is given followed by the administration of the immunoconjugate linked to Yttrium-90 (0.4 mci/Kg). Ibritumomab induces apoptosis in cells expressing CD20 antigen, and the beta emission from Yttrium-90 causes damage to the cells by the formation of free radicals. Since CD20 antigen is not expressed on B-cell precursors, the B cells recover usually in about 9 months after therapy with ibritumomab regimen. The side effects associated with ibritumomab therapeutic regimen include anemia, thrombocytopenia, neutropenia, increased risk of infections predominantly bacterial in nature, hemorrhage, allergic reactions including the bronchospasm, angioedema, gastrointestinal discomfort, and increased cough. There is also some risk (2 %) of secondary malignancies after treatment with ibritumomab regimen.

5.8.5.16 Tositumomab (Bexxar)

The tositumomab therapeutic regimen is also composed of a monoclonal antibody and radioisotope iodine-131. Tositumomab is a murine monoclonal antibody (IgG_{2a}lambda) specific for the CD20 antigen (Fig. 5.6). The covalent linkage of iodine-131 to tositumomab is used to produce iodine-131 tositumomab. The therapeutic regimen of tositumomab causes lysis of the target cells by various mechanisms including apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity. The cell death also results from the ionizing radiation of Iodine-131.

This therapeutic regimen is indicated for the treatment of relapsed or refractory, low-grade, follicular, or transformed non-Hodgkin's lymphoma, all expressing CD20 antigen. It is not an initial treatment of choice but is used in patients who have not successfully responded to standard chemotherapeutic regimen or the combination of chemotherapy and rituximab. Tositumomab therapeutic regimen is used as a single course of treatment and is not used in combination with other chemotherapeutic or radiation treatments. It is administered to patients in two steps, termed as dosimetric and therapeutic, and each step has two components. During the first

component of dosimetric step, 450 mg of tositumomab is administered intravenously followed by the administration of the second component (Iodine-131) tositumomab at a dose of 5.0 mci (Iodine-131 and 35 mg tositumomab) intravenously. The therapeutic step is then followed 7–14 days later, which is composed of an initial administration of 450 mg of tositumomab followed by the intravenous infusion of Iodine-131 tositumomab. The median half-life of tositumomab is 67 h, ranging from 28 to 115 h. Iodine-131 is eliminated by decay and excreted in urine. Sixty-seven percent of the injected dose is cleared in 5 days with 98 % of the clearance in the urine.

The adverse effects associated with tositumomab therapeutic regimen include anemia, thrombocytopenia, neutropenia, infections, hemorrhage, and allergic reactions. Increased risk of secondary neoplasia and myelodysplasia has also been reported with this regimen. Other side effects produced by tositumomab therapy include pneumonia, pleural effusion, dehydration, gastrointestinal discomfort, and infusional toxicity. Delayed adverse reactions include hypothyroidism and HAMA.

5.8.5.17 Brentuximab Vedotin (Adcetris)

Brentuximab vedotin is an antibody drug conjugate directed against CD30 molecules. CD30 (KI-1, BERH2 antigen) is a member of the TNFR SF (tumor necrosis factor receptor superfamily), and the drug binds to the TNFR-associated factor (TRAF) 1, TRAF2, TRAF3, TRAF3, and TRAF5. They are signal transduction molecules associated with TNFR2 and CD40. These molecules induce cell proliferation or cell death. CD30 is expressed in classical Hodgkin's lymphoma and systemic anaplastic large cell lymphoma, but is not expressed on resting blood T cells, B cells, and natural killer cells. However, activation of these cells results in its induction. It is expressed on large cell lymphoma and a number of other lymphocytic cancers. In different cells types, CD30 activates various signaling pathways. The mechanism of action of CD30 is the overproduction of NFκB. This overproduction can be constitutive or a product of the induced expression of CD30. Enhanced levels of soluble CD30 are seen in patients with Hodgkin's lymphoma. These patients carry a high risk of unsuccessful chemotherapeutic treatment. Brentuximab vedotin is composed of a chimeric monoclonal antibody brentuximab, which is specific for CD30 molecules and is attached to an antimitotic agent monomethyl auristatin E via a linker. The peptide monoclonal antibody–drug bond allows a rapid release of the antimitotic agent inside the cancer cell. The Fab region of the monoclonal antibody binds to CD30 on the cell membrane of the cancer cells, delivering tumor-killing drug. After binding to the cell surface of the target cell, brentuximab vedotin is internalized by endocytosis and as a result selectively targets the tumor cells. The monoclonal antibody is used to treat classical Hodgkin's lymphoma and systemic anaplastic large cell lymphoma. Chemotherapy-induced peripheral neuropathy is one of the adverse effects of the drug. The symptoms include sometime irreversible tingling, numbness, severe pain, and hypersensitivity to cold, involving hands, feet, arms, and legs. Other side effects of brentuximab

vedotin are nausea, vomiting, diarrhea, cough, anemia, fatigue, upper respiratory tract infection, fever, rash, thrombocytopenia, and neutropenia.

5.8.6 Monoclonal Antibodies for Other Disorders

5.8.6.1 Eculizumab (Soliris)

Eculizumab is a recombinant humanized monoclonal IgG2/4 (IgGκ) antibody, which is first in its class. It is an inhibitor of the terminal step of complement cascade (Fig. 5.9). Eculizumab binds to terminal complement component 5 (C5), which when activated kills invading pathogens as well as some host cells by creating pores, and is also involved in recruiting pro-inflammatory cells. C5 acts in the late stage of the complement cascade. The drug is used for the treatment of paroxysmal nocturnal hemoglobinuria (PNH). PNH is a rare disorder, presented by enhanced destruction of red blood cells and abnormal blood clotting. In some cases, the disorder could potentially be fatal. Eculizumab is also used to treat atypical hemolytic uremic syndrome (aHUS), which is also a blood clotting disorder and a rare genetic disease. The drug is being assessed for the treatment of neuromyelitis optica, membranoproliferative glomerulonephritis (MPGN), dense-deposit disease (DDD), cold agglutinin disease, and catastrophic antiphospholipid syndrome

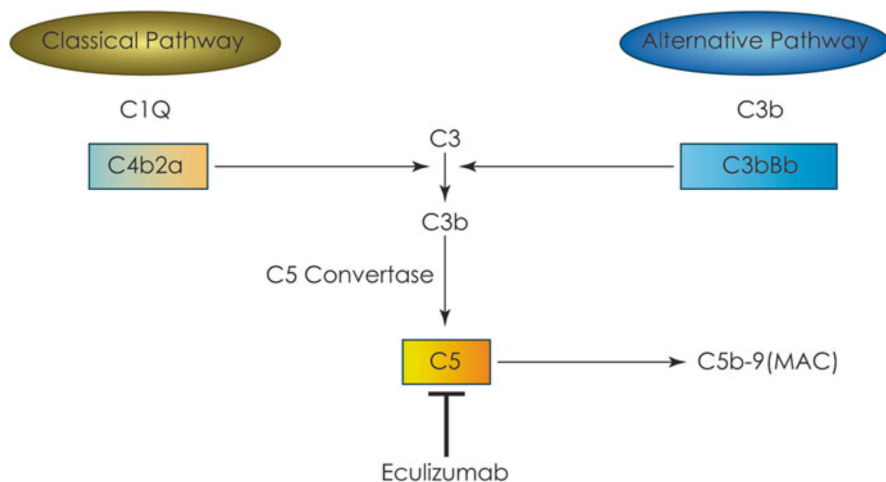


Fig. 5.9 Mechanism of action of eculizumab: Eculizumab is directed against the terminal steps of the complement cascade. Specifically, it binds to the terminal complement component 5 (C5) by inhibiting the cleaving of C5 to C5a and C5b by the C5b convertase. These actions block the later stages of complement cascade. C5 after its induction plays a role in inflammatory immune response. Paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome benefit from the use of this monoclonal antibody by decreasing the need of blood transfusions and increase in the renal function in the latter case

(CAPS). The adverse effects associated with eculizumab include headache, nausea, fatigue, nasopharyngitis, cough, upper respiratory infections, and back pain.

5.8.6.2 Denosumab (Prolia, Xgeva)

Denosumab is a fully human IgG2 monoclonal antibody directed against receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL is also known as tumor necrosis factor ligand superfamily member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE), osteoclast differentiation factor (ODF), or osteoprotegerin ligand (OPGL). It is a protein that serves as a primary signal for bone removal. Bone remodeling is a normal physiological function where the old bone is replaced by the new bone. Several types of cells including osteoblasts, osteoclasts, and osteocytes play a role in this process. Osteoblasts secrete new bone, whereas osteoclasts dissolve old bone. The receptors for the activator of nuclear factor kappa-B (RANK) are expressed on the precursors of osteoclasts, which are called pre-osteoclasts that is a member of tumor necrosis receptor superfamily. Their ligand RANKL induces the maturation of pre-osteoclasts to osteoclasts. Denosumab binds to its antigen RANKL and neutralizes its effects on the conversion of pre-osteoclasts to osteoclasts. In addition, inhibition of RANKL allows osteoprotegerin to reduce the production of osteoclasts. Osteoprotegerin is a decoy receptor for RANKL. The patients suffering from osteoporosis have lower levels of osteoprotegerin.

Denosumab (Prolia) is used to treat osteoporosis, whereas denosumab (Xgeva) is used to treat bone damage due to bone metastasis from solid tumors. The most adverse side effects of the antibody include back pain, skin problems, pain in extremity, hypercholesterolemia, cystitis, musculoskeletal pain, hypocalcemia, infection, nausea, diarrhea, sore throat, runny nose, and allergic reactions.

5.8.6.3 Ranibizumab (Lucentis)

Ranibizumab is a humanized FAB fragment directed against vascular endothelial growth factor A (VEGF-A). The fragment is the same as in bevacizumab and is an inhibitor of angiogenesis. It inhibits the formation of new blood vessels under the retina. The drug is used to treat a “wet” type of age-related macular degeneration (AMD), macular edema following retinal vein occlusion (RVO), and diabetic macular edema (DME). The “wet” type of age-related macular degeneration (AMD) is age-related vision loss. Ranibizumab is administered intravitreally once a month. If that is not possible, it could be injected every 3–4 months. The most common eye-related adverse effects include intraocular inflammation, vitreous floaters, conjunctival hemorrhage, eye pain, and increased intraocular pressure. The risk of arterial thromboembolic events is low which is generally associated with intravitreal injection of VEGF inhibitors. The injection procedure has a potential of causing retinal detachment, traumatic cataracts, and endophthalmitis, though their incidences have been negligible.

5.8.6.4 Omalizumab (Xolair)

Omalizumab is a recombinant glycosylated humanized monoclonal antibody (IgG1k) directed against human IgE. It binds to free IgE in the blood and interstitial fluid. It does not bind to IgE which is bound to its receptors (FcεRI) on effector cells, but binds to membrane-bound IgE. The effector cells include mast cells, basophils, and dendritic cells. Omalizumab interferes with the binding of IgE to its high affinity receptors (FcεRI). By steric hindrance, it also inhibits the binding of IgE to FcεRII. The antibody is used to treat moderate to severe form of allergic asthmatic patients who do not respond well to high doses of glucocorticoids. It is also being used to treat chronic spontaneous urticaria and chronic idiopathic urticaria. Omalizumab is injected once every 2 or 4 weeks subcutaneously. The serum levels of IgE and the body weight of the patient are the determinants of the frequency of the dosing regimen. The side effects include anaphylaxis, heart disease, strokes, and in some cases Churg–Strauss Syndrome (who have underlying eosinophilic disorder).

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Chapter 6
Allergic Disease

Abstract This chapter focuses on the etiology, pathogenesis, and immunotherapeutic strategies for the allergic disease. In atopic individuals IgE is produced after exposure to low levels of environmental or food allergens instead of IgG. The first part of this chapter is devoted to defining hypersensitivity disease, IgE-mediated responses, and regulation of IgE synthesis. IgE synthesis is regulated by heredity, natural history of antigen exposure, nature of antigen, helper T cells, and cytokines. This is followed by a discussion of hygiene hypothesis in the etiology of allergic disease and mechanism of allergic inflammation. T cells, cytokines, and cytokine-dependent signaling pathways play a crucial role in the development and pathogenesis of the disease. In this regard the roles of IL-4, IL-5, IL-9, and IL-13 along with IL-4/STAT6 signaling, Notch-signaling pathway, and IL-2/STAT5 pathway are discussed. The applications of immunotherapy and sublingual immunotherapy for traditional allergy as well as allergy to food and seminal plasma are described.

Keywords Atopy • Hypersensitivity • Type I reactions • Type II reactions • Type III reactions • Type IV reactions • Type V reactions • Antibody-dependent cell-mediated cytotoxicity • Complement • Histamine • Leukotrienes • Prostaglandins • Mast cells • Allergic rhinitis • Allergic asthma • Atopic eczema • Anaphylaxis • Urticaria • IgE • Eosinophils • Chemotaxis • TCR • HLA • TH2 cells • IL-4 • IL-5 • IL-9 • IL-13 • TH1 cells • Wheal and flare reaction • Hygiene hypothesis • STAT-6 • GATA3 • Treg cells • Nuclear factor κB • Dendritic cells • Helper T cells • Cysteinyl leukotrienes • TNF-α • $\alpha_4\beta_1$ integrin • Vascular adhesion molecules • Eotaxin-1 • Eotaxin-2 • Eotaxin-3 • Monocyte chemotactic protein 3 and 4 • RANTES • TARC • T-bet • Addressin • Integrin • T-cell homing • IL-1 • CCR8 receptors • TLR2 • TLR4 • SPA • IL-8 • IL-1β • Thymic stromal lymphopoietin • IL-7 • $CD45RO^+$ • CCR • STAT3 • STAT5 • Foxp3 • Eotaxin-1 • Eotaxin-3 • P-selectin • IL4/STAT6 signaling • Notch-signaling pathway • IL-2/STAT% pathway • mTORC2 • TCF-1 • Immunotherapy • Sublingual immunotherapy • Oralair • RAGWITEK • Isotype switching • Food allergy • Mucin • IgA • Gut-associated lymphoid tissue • Basophils • Seminal plasma allergy

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6.1 Introduction

The term allergy (from Greek allos, meaning "other," and ergon, meaning "work") was introduced in 1906 by von Pirquet. Atopy from Greek meaning "out of place" is also used to describe allergic disease. Following a blood transfusion, a physician in 1919 first reported allergy-related symptoms of transient asthma. In the study a response to horse dander suggested that an allergic reaction could be mediated by a factor in the blood. The passive transfer of a positive skin test performed by Prausnitz and Kustner, in an experiment in 1921, led to the search of substances responsible for allergic reaction. Until 1960 the focus was on several labile complexes in the serum rather than one molecule, and it was assumed that antibodies were not involved in allergic reaction.

In 1965, Bennick and Johansson in Sweden reported a new class of immunoglobulin that they called IgND, and it was subsequently found that the levels of IgND were several-fold higher in allergic asthmatics as opposed to normal subjects. A new immunoglobulin was also identified by the Ishizaka's group in the United States in 1966–1967 when they found that an antiserum could suppress the allergic reaction; it was termed "YE-globulin" although they were unable to isolate it due to its very low levels in serum. In 1967, it was determined that IgND and YE-globulin were the same molecules and were given a new name, IgE.

6.2 Hypersensitivity Disease

The human immune response protects the host by maintaining homeostasis. The immune system is activated as a result of exposure to antigen, allergen, infection, and/or neoplasm, resulting in the alteration of homeostasis. In some instances, an immune response generated against a foreign invader is harmful to the host itself, which is referred to as hypersensitivity disease which has been divided into five subtypes:

Type I reactions: These reactions result from the production of IgE; termed as immediate hypersensitivity disease. These are allergic reactions that emanate from re-exposure to allergens where their binding to IgE results in the degranulation and secretion of endogenous mediators including histamine, leukotrienes, prostaglandins, and cytokines from sensitized mast cells. These products cause vasodilation and smooth muscle contraction. These reactions vary from local to systematic producing symptoms from mild allergic reaction to rare anaphylactic shock resulting in death. The clinical examples of type I reactions include allergic rhinitis, allergic asthma, atopic eczema, anaphylaxis, and urticaria.

Type II reactions: These reactions result from the recognition of cells to which antigens (extrinsic or intrinsic) are bound by macrophages or dendritic cells resulting in a B-cell response and production of the antibodies against foreign antigens. They also involve antibody-dependent cell-mediated cytotoxicity, and complement plays a role in this process as well. The reactions result from the

hemolytic disease of the newborn as well as adverse effects to some drugs, specifically those causing hemolytic anemia. Other clinical examples include transfusion reactions, autoimmune hemolytic anemia, immune thrombocytopenia, pernicious anemia, and rheumatic fever.

Type III reactions: These reactions involve the presence of antigen–antibody complexes, particularly those formed as a result of the production of autoantibodies. These complexes deposit in various tissues and involve inflammatory cells as well as the complement, resulting in tissue damage due to the production of proteolytic enzymes by polymorphonuclear leukocytes and macrophages. A number of autoimmune diseases result from these reactions. Some clinical examples include systemic lupus erythematosus, rheumatoid arthritis, immune complex glomerulonephritis, Arthus reaction, and serum sickness.

Type IV reactions: Also termed as delay-type hypersensitivity reaction, they take 48–72 h to develop and are not antibody mediated. Antigens are recognized by $CD4^+$ and/or $CD8^+$ cells in context of MHC class restrictions on antigen-presenting cells. These reactions are T-cell mediated where activated T cells release cytokines, resulting in the development of granulomas from macrophages. These mechanisms are responsible for symptoms which may include transplant rejection, contact dermatitis, leprosy, tuberculosis, and sarcoidosis.

Type V reactions: This reaction is occasionally used as separate from type II reactions. In this case, antibodies bind to the cell surface receptors instead of cell surface components, resulting in the impairment of cell signal either via augmentation or suppression. Some clinical examples include myasthenia gravis and Graves' disease.

6.3 IgE-Mediated Responses

IgE, one of the five isotypes of antibodies made by humans, was designed to be an anti-parasitic immunoglobulin. Helminths results in a strong IgE response, which also include the production of parasite-specific IgE antibody. The beneficial effects of IgEs include roles of early recognition of invading organisms and allergens and stimulation of immunity due to better antigen presentation. IgE could expel allergenic materials from the body since typical allergic reaction produces sneezing, coughing, mucus, tears, bronchoconstriction, inflammation, and even vomiting. Nonetheless, the production of IgE instead of IgG in response to an antigen contributes to the etiology/pathogenesis of allergic disease. IgE binds to cells via high-affinity or low-affinity Fc receptors. Mast cells primarily express high-affinity receptors, whereas eosinophils, lymphocytes, and a variety of other cell types express low-affinity receptors. An allergic reaction has three fundamental features:

1. Synthesis of IgE specific to an allergen
2. Initial binding of allergen-specific IgE to high-affinity Fc receptors on mast cells followed by second exposure to the same antigen, which causes cross-linking of

pairs of IgE molecules, resulting in the activation and degranulation of mast cells, which produces the signs and symptoms of immediate hypersensitivity reaction

3. Allergic inflammation resulting from the recruitment and activation of eosinophils

These processes depend on chemotactic activation of the effector cells and altered cellular migration to promote an allergic reaction. During these events, there is also an upregulation of the adhesion molecules, both on the effector cells and on high endothelial venules in blood vessels, lungs, and other tissues.

In atopic/allergic subjects, IgE is produced following contact with low levels of environmental allergens. The immune response begins with sensitization, after an allergen such as pollen, animal dander, or house dust mite is inhaled, and the allergen is processed by antigen-presenting cells such as Langerhans cells. These cells, which are present in the epithelium linings of the lungs and nose airways, uptake, process, and present the processed antigen to the TCR in context of HLA molecules. Activated T cells secrete cytokines that along with other cell–cell interactions transform B cells to plasma cells, which are the antibody-secreting cells. In allergic disease, plasma cells produce IgE instead of IgG that could bind to its specific allergen via its Fab region. The IgE binds to the Fc receptors on the cells and this phase of immune response is called sensitization phase (Fig. 6.1).

After re-exposure to the same allergen, a robust and rapid memory response is initiated. Signal transduction emanating from the cross-linking of IgE antibodies bound to mast cells by allergen results in the degranulation and release of mast cell mediators. Mast cells also regulate the expression IgE receptors in order to maintain a given number of unoccupied receptors. It is feasible that this phenomenon is regulated by the levels of the circulating IgE.

We are all (atopic or nonatopic) exposed to aeroallergens which include pollens, house dust mites, and dander. The nonatopic individuals mount a weak immune response to the allergens by producing the antigen-specific IgG_1 and IgG_4 antibodies, whereas the atopic individuals produce allergen-specific IgE antibodies. T cells from atopic individuals respond to the aeroallergens against which they were generated by elaborating TH2 cytokines including IL-4, IL-5, and IL-13. The infiltration of TH2 cells into the affected tissues is a hallmark of the allergic disease and asthma.

The common environmental allergens which cross the placenta prime the T cells of the fetus and all of the newborns have predominantly TH2 cells. As the infant grows, this composition shifts to TH1 cells and TH1-mediated responses against aeroallergens. However, in atopic infants there is further development of TH2 cells and TH2-mediated responses. Epidemiological studies on families, particularly on twins, have suggested that IgE production is due to genetic predisposition. This is based on their ability to produce more TH2 cells and subsequently IL-4-dependent immune response to aeroallergens.

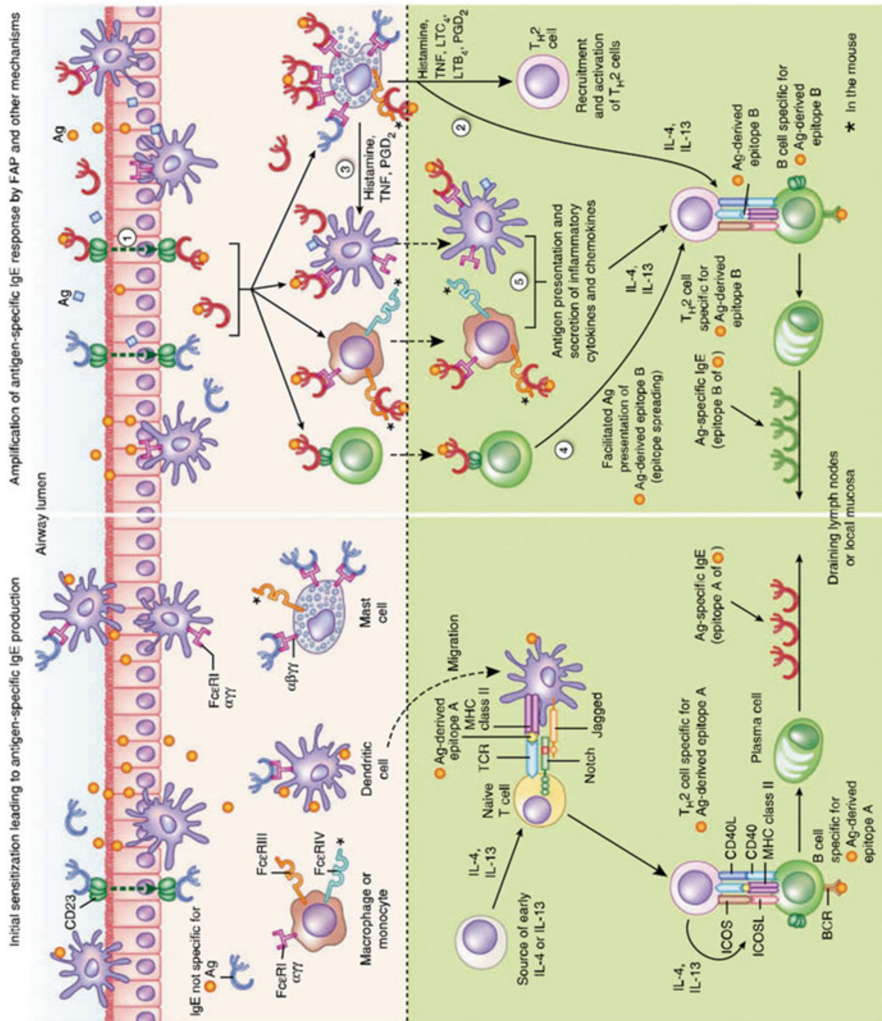

Fig. 6.1 The sensitization to allergen and production of IgE: the allergens are uptaken by dendritic cells or macrophages in the epithelium of the airway mucosa or in the airway lumen or reach the submucosal dendritic cells as a result of disruption to the epithelium. The dendritic cells are activated by the allergens and enter the lymph nodes to present processed allergen to T cells, in the presence of IL-4 and IL-13, resulting in the differentiation of T cells to TH2 cells. This ultimately results in the production of allergen-specific IgE. FAP and other mechanisms augment allergen-specific IgE production. Allergen-specific IgE binds to the mast cells via FcεRI receptors and its re-exposure to the allergen causes degranulation and secretion of endogenous mediators. CD23 on airway epithelial cells allows crossing of IgE and allergen–IgE complexes across the epithelium. This results in the binding of IgE and allergen–IgE complexes to mast cells and dendritic cells and their activation, which is the contributing factor for allergic inflammation. IL-4 and IL-13 are required for the production of inflammation. A process called FAP results in epitope spreading. This allows the production of IgE which recognizes new epitopes on the same allergen through their presentation to T cells and the proliferation of TH2 cells in the presence of IL-4 and/or IL-13. There is subsequent production of new allergen-specific IgE against these epitopes. ICOS are inducible T-cell costimulators which are immune checkpoint proteins, expressed on T cells and important for Th2 cells (Reproduced with permission. Source: Galli SJ, Tsai M (2012) Mast cells in Allergic disease. *Nature Med* 18: 693–704. Nature Publishing Group)

6.4 Regulation of IgE Synthesis

The IgE production between atopic and nonatopic subjects is critically different. The nonatopic individuals normally synthesize IgM and IgG and only small amounts of IgE, whereas atopic individuals produce high levels of IgE in response to aeroallergens. The synthesis of IgE is regulated by four interacting factors: heredity, the natural history of antigen exposure, the nature of the antigen and helper T cells, and their cytokines. It has been reported that atopy runs in families and atopic individuals have high levels of IgE. MHC Class II molecules play a role in antigen presentation. In one example, different Class II molecules may serve to present ragweed antigen. Atopic reaction develops as a result of repeated exposure to a particulate antigen. Consequently, a change in geographical location often benefits patients with allergic rhinitis or asthma. The influence of natural history of exposure to an antigen seen with bee sting is the most dramatic example. First exposure does not do anything but second exposure could be potentially fatal.

Allergens that produce strong immediate hypersensitivity reactions are proteins or chemicals bound to proteins. It has not been established why some antigens produce strong allergic reactions while others do not. It is feasible that the property of being allergenic may be due to the nature of the antigen itself, and the epitopes seen by T cells may contribute to this effect. Alternatively, some protein antigens interact with adjuvants naturally which favor IgE synthesis. A number of allergens induce proteolysis, since they are enzymes in their natural state and their enzymatic properties render allergenic activity. Aerodynamic properties also result in allergenic activity depending on the size of the particles. In the Unites States, the major allergens include Der p1 and Der p2 (house dust mites); Fel d1 (cat); Amb a1, 2, 3, 5, and 6 (short ragweed); Amb t5 (giant ragweed); Phl p1 and Phl p5 (timothy grasses); and Bet v1 (birch tree). Other serious allergens include ara H 1, 2, and 3 (peanuts) and Hev b 1,2,3,4,5,6, and 7 (latex).

6.5 Immediate Hypersensitivity Reaction

Allergen-dependent immune response constitutes two phases. The initial response, which occurs within 15 min of recognizing the allergen, is called the early-phase reaction, or immediate hypersensitivity. Four to 6 h after the termination of the symptoms of the first phase, the second or late phase starts, which may persist for days or up to weeks. The mast cells release autacoids including histamine, leukotrienes, prostaglandins, and thromboxane during the early-phase reaction. The local responses produced by autacoids include sneezing, itching, runny nose, edema, mucus secretion, nasal congestion, and bronchoconstriction. The symptoms of the late-phase reaction include cellular infiltration, fibrin deposition, and tissue destruction in lungs. With continued allergic responses, this results in allergic hyperreactivity response, including edema and serious inflammatory response (Fig. 6.2).

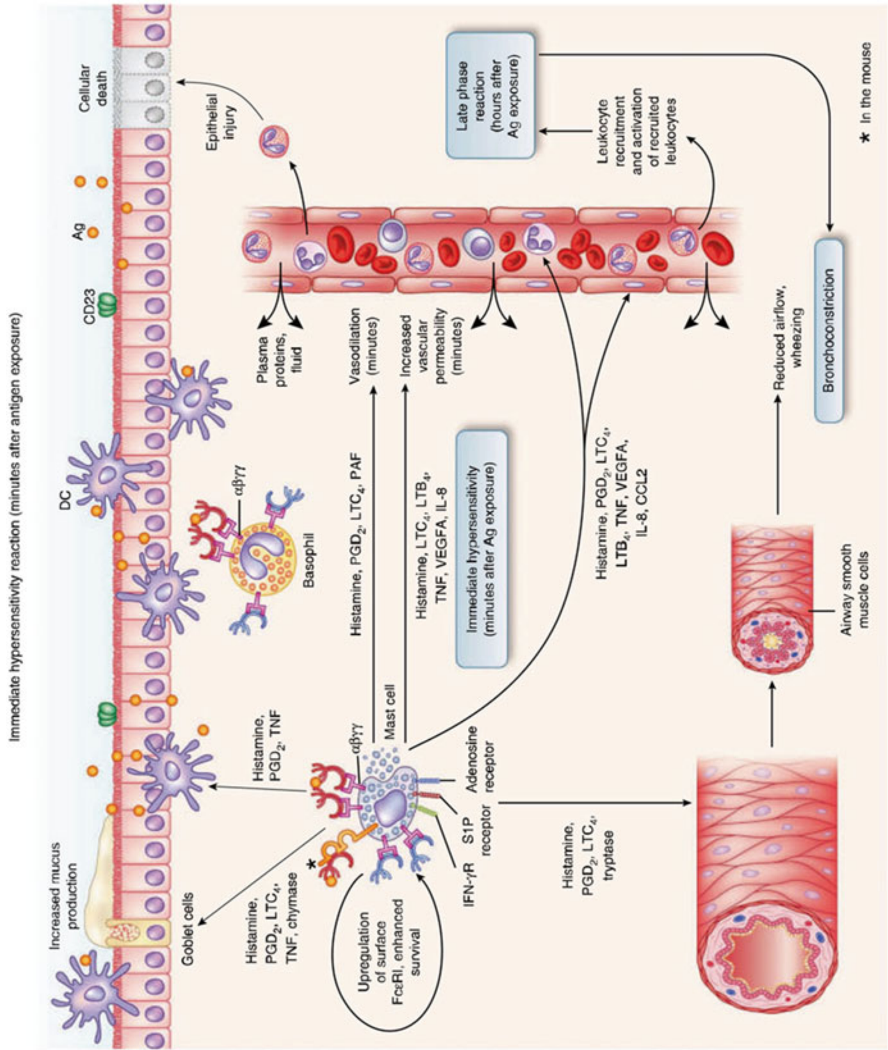

Fig. 6.2 The immediate hypersensitivity reaction minutes after allergen exposure: the binding of IgE to its receptors upregulates the expression of these FcεRI. The immediate hypersensitivity response commences after recognition of allergen by FcεRI, resulting in the secretion of autacoids including histamine, leukotrienes, PGD2, various cytokines, and chemokines from mast cells. The collective effects of endogenous mediators include rashes, inflammation, smooth muscle contraction, bronchospasm, increased vascular permeability, vasodilation, increased production of mucus asthma, and in severe cases anaphylactic shock, which may even cause death. In allergic responses TH2 cells are important in recognizing allergens in context of MHC molecules and secrete IL-4, IL-5, and IL-13. IL-4 induces isotope switching from IgG to IgE and IL-5 is involved in eosinophil recruitment. IL-8 serves as a chemical signal to attract neutrophils at the site of inflammation. These endogenous mediators also impact dendritic cells that results in their migration and maturation. These events contribute to late-phase reaction. There is an influx of circulating leukocytes and upregulation in the expression of adhesion molecules on high endothelial venules. This is also accompanied by production of chemotactic mediators and chemokines. The cumulative effect is the bronchoconstriction and severe inflammation during the late-phase reaction (Source: Galli SJ, Tsai M (2012) Mast cells in Allergic disease. *Nature Med* 18:693–704. Nature Publishing Group, Reproduced with permission)

The classical example of immediate hypersensitivity in humans is the "wheal and flare" reaction. Challenging a sensitized individual with an intradermal injection of an allergen results in redness at the injection site. This is due to locally dilated blood vessels engorged with red blood cells. During the second phase, the leakage of plasma from the venules will result in a rapid swelling of the injection site. The soft swelling is called a wheal and the area of the skin involved in this soft swelling could be as large as several centimeters in diameter. In the third phase, there is dilation of the blood vessels at the margins of the wheal. The blood vessels become engorged with red blood cells which produce a flare, the characteristic red rim. It takes about 5–10 min after the administration of the allergen for the development of full wheal and flare reaction and it lasts less than an hour. There is a slight separation of the endothelial cells in the venules of the wheal as observed by the electron microscopy. This separation results in the escape of macromolecules and fluid, and there is a release of preformed mediators by mast cells in the area of wheal and flare.

In nonatopic infants, according to the hygiene hypothesis, the main stimuli for TH1-dependent immune response are the microbes. Macrophages not only secrete IL-12 but also engulf the microbes, and IL-12 induces TH1 cells and natural killer cells. The stimulated TH1 cells and natural killer cells secrete interferon-γ which results in a nonatopic state where an individual is protected from the allergic disease. It has been suggested that the lifestyle in the West may be responsible for an increase in the prevalence of allergic disease in the developed industrial states. This could be due to an absence of microbial antigens which stimulate TH_1 cells. The contributing factors could be a comparatively clean environment and a widespread use of antibiotics in early life.

The hygiene hypothesis was initially proposed by David Strachan in 1989 who observed that allergic disease was less common in children from larger families than in children from families with only one child. This was attributed to exposure to more infectious agents among children belonging to larger families. According to the hygiene hypothesis, in families with only one child, insufficient stimulation of TH1 responses which are elicited by bacterial and/or viral infections results in a robust TH2 response, secreting high levels of IL-4, IL-5, and IL-13 and consequently leading to allergic disease. The atopic status depends on the environment, beginning in the early childhood. Children living on farms or with multiple pets, which exposes those to high levels of endotoxins, have lower incidence of allergic disease. The exposure to animal sheds and haylofts and the consumption of unpasteurized cow's milk play an important role in this regard. Furthermore, the timing of exposure is critical since the protection was the best when the exposure took place during the first years of life as opposed to the later years. The high exposure to endotoxins promotes the development of IFN-γ-secreting TH1 cells, which inhibits the development of TH2 responses. Three aspects of hygiene hypothesis have been proposed to overcome the criticism of this hypothesis, particularly the observations that there has also been an increase in the incidence of autoimmune diseases such as multiple sclerosis, Crohn's disease, type I diabetes, and inflammatory bowel disease, which are associated with overactive TH1 response in the same patients with

increased allergic disease. These three possibilities include overt and unapparent bacterial and viral infections, noninvasive exposure to microbes in the environment, and the effects of infections on innate and acquired immune responses including the development of regulatory T cells.

The mechanisms are complex since timing of the exposure to allergen, state of development, and prenatal factors acting either in utero or modulating subsequent development may all play some role in the development of the allergic disease. New data suggest that the interactions between the environment and genes may be the result of multiple genes responding to the environment at several stages of development rather than a single gene, ultimately leading to the etiology/pathogenesis of allergic disease.

Some observations have suggested a role of the presence of the microbial compounds in the environment which may affect hosts' immune response. For example, the children of farmers who are exposed to high levels of endotoxins exhibit increased expression of TLR2 and CD14 as opposed to children with less exposure. This suggests a modulation of innate immune response in the absence of an infection. Activation of TLR induces inflammatory and acquired immune response, and inhibition of one of these pathways (MyD88) results in decreased TH1 responses and enhanced IgE production.

To respond to the criticism of an increased prevalence of both the autoimmune and allergic disease, an alternative mechanism has been proposed. According to this hypothesis, a lack of stimuli from infectious agents during development results in poor development of regulatory T cells. A poor regulatory T-cell function increases the risk of the development of an autoimmune response due to inability to suppress TH1 function, which may also lead to an enhanced TH2 response.

The microbes by producing an environment rich in IL-12 could drive TH1-mediated responses. The production of IgE is induced by IL-4 and IL-13, which initiate transcription of the gene for a particular region of the IgE heavy chain ε class of the constant region. STAT-6 and NFκB, two transcription factors, are also required for the production of IgE. IL-4 activates STAT-6, and nuclear factor κB pathway needs costimulatory molecule CD40 and its ligand CD154. Furthermore, the presence of the transcription factors GATA-3 and C-maf favor TH2 cell-mediated allergen-specific responses. The TH2 paradigm suggests that the etiology and pathogenesis of allergic disease involves a complex interaction of T cells, B cells, and antigen-presenting cells, resulting in higher production of IL-4 and IL-13, lower production of INF-γ, and elevation of IgE.

Although the mechanisms related to TH1/TH2 balance may be pivotal in explaining the etiology of allergic disease, they may not be sufficient. For example, serum levels of IgE are also dependent on the factors regulating terminal differentiation of class-switched B cells and the rate of IgE secretion, in addition to isotype switching alone. This process is regulated by IL-6. Furthermore, regulatory T cells and their interaction with dendritic cells both play an important role in the regulation of the development of allergic disease.

In addition to hygiene and allergic paradigms, epithelial and viral paradigms have also been proposed to explain the increases in atopic disorders. It seems that in

addition to $CD4^+$ cells, a number of other cell types including the $CD4^+CD25^+$, dendritic cells, and others may be involved in the pathogenesis of allergic disease. Nonetheless, it is difficult to imagine a strong allergen-driven allergic/asthmatic response if helper T-cell activation is shut down. Consequently, developmental dysregulation of helper T cells ($CD4^+$) in childhood is important in the pathophysiology of allergic/asthmatic disease.

6.6 Allergic Inflammation

The immediate hypersensitivity reaction is followed by the late-phase reaction that is manifested by red, edematous, and indurated swelling in the skin, blockage in the nose, and wheezing in the lungs. Acute allergic reactions are the result of immediate hypersensitivity, and the mediators released by the mast cells produce its clinical symptoms, including anaphylaxis, rhinoconjunctivitis, and urticaria. Three cysteinyl leukotrienes, C_4, D_4, and E_4, are produced by mast cells, and their pathological effects include smooth muscle contraction, vasodilation, hypersecretion of mucus, and increased vascular permeability. The granules of mast cells also contain a protease, tryptase, which activates receptors on endothelial and epithelial cells resulting in a number of processes which also include an enhanced expression of adhesion molecules. The role of histamine in asthmatic disease has not been fully elucidated although the new generation of antihistamines has shown some benefits in childhood asthma.

The activation of mast cells and T cells result in late-phase reactions. Both immediate hypersensitivity and late-phase reactions are evident in the skin of atopic as well as nonatopic individuals after cross-linking of IgE-bound mast cells with an antibody against IgE. The atopic asthmatic could develop late-phase reaction even in the absence of mast cell-related immediate hypersensitivity reaction, which is mast cell independent and HLA dependent, suggesting the role of T cells by themselves in causing asthma symptoms in atopic asthmatics.

Antigen-presenting cells, specifically cutaneous Langerhans cells and dendritic cells, are important in atopic eczema and asthma, respectively. These antigen-presenting cells are responsible for presenting the processed allergen to T cells in an HLA-restricted manner. This process is further stimulated by the presence of GM-CSF in the airways of atopic asthmatics. GM-CSF also serves as a stimulus for macrophage production, which presents antigen to helper T cells with a resultant increase in the development of TH2 cells and the elaboration of their cytokines in atopic asthmatic patients. The most important cytokine associated with allergic inflammation and released by TH2 cells include IL-4, IL-5, and IL-13. IL-9 secreted by TH9 cells is also involved in allergic inflammation. The stimulation of IgE production is the most important role of IL-4 and IL-13, which also augment the expression of vascular cell adhesion molecule 1. The development of mast cells is regulated by IL-4 and IL-9, whereas the production and differentiation of eosinophils is regulated by IL-5 and IL-9. The airway hyperresponsiveness is dependent

on IL-9 and IL-13 and the production of mucus is regulated by IL-4, IL-9, and IL-13.

Cysteinyl leukotrienes and platelet-activating factors released by eosinophils injure mucosal surfaces of the airways. Furthermore, they damage M_2 muscarinic receptors, and as a result, the cholinergic response is unchecked in the absence of inhibitory M_2 muscarinic receptors. The production of eosinophil is regulated by IL-5, which also regulates their terminal differentiation. The preferential accumulation of eosinophils in the airways of the atopic asthmatics results from a combination of interaction between adhesion receptors, chemokines, and cytokines. The interacting molecules include $\alpha_4\beta_1$ integrin, vascular adhesion molecules, eotaxin-1, eotaxin-2, eotaxin-3, monocyte chemotactic protein 3 and 4, RANTES, GM-CSF, IL-3, and IL-5. Allergic inflammation also results from several neuropeptides including substance P, neurokinin A, and calcitonin-gene-related peptide. Furthermore, thymus and activation-related chemokine (TARC), a ligand for the foregoing TH2 cells, is upregulated in bronchial epithelial cells in allergen-challenged asthmatics. Consequently, TARC represents a molecule involved in allergen-induced asthma. Production of IL-1 and TNF-α leads to the induction of transcription factor NF-κB and TARC expression. TARC expression results in the recruitment of TH2 cells, where it is a ligand for the chemokine receptor CR4 that is expressed on TH2 cells.

Tissue-specific transcription factors, T-bet, GATA-3, and CMAF, regulate helper T-cell differentiation, and genetic defects in these factors may alter the TH1/TH2 balance. However, paradigms other than the TH1/TH2 imbalance have also been proposed for the etiology of allergic/asthmatic disease. Allergic inflammation may also occur through innate immune stimulation, which is not directly related to TH1/TH2 dichotomy. TNF-α may be involved in the inflammatory response in adults with asthmatic disease, and as a consequence, inhibition of TNF-α may be useful in severe asthma. IL-10 also plays an interesting role in this process; while being produced by both regulatory T and TH2 cells, it may downregulate early-life inflammation, however, and in other situations it could serve as a TH2 pro-inflammatory cytokine depending on its cellular origin.

It has been suggested that the increased risk of allergic inflammation may result from T-cell dysregulation. This could result from either a global lack of suppression of pro-inflammatory cytokine production or by the upregulation of TH2 response. Regulatory T cells participate in the induction of immune tolerance. $CD4^+CD25^+$ regulatory T cells play a specific role in protection against allergic disease and asthma.

6.7 T Cells Homing to the Epithelial Barriers

Allergic sensitization is generally the result of a defect in the epithelial barrier resulting in the abnormal activation of the epithelium. Environmental as well as host factors contribute to these anomalies. Changes in the barriers in the skin and the

lung contribute to the activation of the epithelium by allergens and subsequent production of cytokines favoring a TH2 phenotype. There is also activation of dendritic cells due to the dysfunctional epithelial barrier, resulting in an environment that produces allergen-specific TH2 responses. The effector T-cell function requires tissue-specific trafficking which is efficient and accurate. The adhesion molecules including integrins, selectins, and chemoattractant receptors play a role in this trafficking. Naïve, effector, and memory T cells follow various migration patterns which are unique for each cell type. As the T cell differentiates from the naïve cell, there are changes in the types of trafficking receptors. Some of these changes are retained by memory T cells after recovery from inflammation. When naïve T cells are originally primed, organs which are susceptible to allergy and interact with the environment allow entry of the inflammatory effector and T cells from the circulation to efficiently serve for immune surveillance. This is the result of programming or imprinting of allergy-prone organs for specific inflammatory effector T and memory T cells. It has been suggested that during immune inflammation and immune surveillance, the skin, lung, and gut become susceptible to developing allergic disease due to preprogrammed organ-specific T-lymphocyte migration pathways. For example, lung- and nasal-associated lymphoid tissues are responsible for programming T cells for organ-specific homing. The nasal-associated lymphoid tissue in its endothelial cell lining exhibits enhanced number of adhesion molecules, addressins, which are a ligand for CD62L. The mucosal-associated tissues do not express these adhesion molecules. Binding to peripheral node addressin after the migration of programmed allergen-specific T cells into nasal-associated lymphoid tissue results in an environment that is conducive for the development of allergic rhinitis. A role of a number of homing trafficking receptors and their ligands has been implicated in atopic dermatitis, asthma, and food allergy. This role of leukocyte trafficking in allergic disease suggests that inhibiting tissue- and inflammation-specific T-cell migration should be therapeutically relevant to treat allergic disease/asthma.

6.8 Receptors Associated with Allergic Disease

IL-1 receptors, Toll-like receptors, and CCR8 receptors are linked to the pathogenesis of asthmatic disease. IL-1 induces the proliferation of TH2 cells, specific antibody responses, and expression of exotoxin as well as eosinophil chemoattractants. It may play a role in T-cell priming through induction of CD4OL and OX40 receptors. Since bacterial infections mount TH1 responses through TLR, a reduction in TLR ligands on bacteria coupled to allergen exposure can cause an inhibition from TH2 to TH1 responses. Activation of TLR-7 provides protective effects against the development of experimental asthma, and both IL-10 and IL-12 are required for the mediation of these effects. TH2 and TH1 cells exhibit differential receptor expression. In neutrophilic asthma, there is an upregulation of the innate immune response and increased expression of TLR2, TLR4, SPA, IL-8, and IL-1β.

6.9 Genetic Predisposition

Atopy runs in families, and various genetic and phenotypic polymorphisms have been correlated in population studies with the onset and development of allergic disease/asthma. There is a correlation of atopic sensitization in atopic children who have asthma at age 7 when compared with children without asthma. The chromosomal regions, which are linked to allergic disease/asthma include 2q33, 5p15, 11p15, 17q11.1, 19q13, and 21q21. Furthermore, chromosomal region 14q contains several polymorphic markers and TAP-1 (transporters associated with antigen processing) – Acc 1 allele polymorphism is associated with atopic bronchial asthma. TAP is found in chromosomal region DQB1 and DRB1 and helps translocate peptides to MHC I surface glycoproteins.

6.10 The Role of T Cells in Allergic Disease

Different types of T cells respond to allergens including $CD4^+$, $CD8^+$, and NKT cells. In allergic responses, TH2 cells are vital in recognizing the allergens in context of MHC Class II molecules as they produce their effector function via elaboration of IL-4, IL-5, and IL-13. The isotype switching from IgG to IgE production is induced by IL-4, and as IL-5 recruits eosinophils, both lead to the etiology/pathogenesis of allergic disease/asthma. Allergic inflammation and its clinical symptoms emanate from various immune pathways orchestrated by multiple cytokines produced by TH2 cells. It has not yet been established why allergens preferentially promote TH2 responses in atopic individuals. The patients with seasonal allergies possess allergen-specific memory T cells. One distinguishing characteristic of memory T cells is the expression of the CD45RO isoform as opposed to CD45RA that is expressed on naïve T cells. They are further classified into effector memory T cells ($CCR7^-$) and central memory T cells ($CCR7^+$), both of which have different functions. Selective expression of a gene encoding chemoattractant receptor-homologous molecules expressed on TH2 cells (CRTH2) has also received attention in order to search for markers exclusively expressed on TH_2 cells. CRTH2 is a prostaglandin D2 receptor present on approximately 0.5 % of memory $CD4^+$ T cells ($CD45RO^+$). These cells specifically secrete IL-4, IL-5, and IL-13 in response to stimulation of the TCR. Allergen-specific recall responses to these cells require priming of dendritic cells by thymic stromal lymphopoietin (TSLP) which is an IL-7-like cytokine.

Although a role of TH2 cells has been well established in allergic disease, the contribution of other types of T cells in this process is under investigation. The presence of gamma–delta T cells play a role in allergic disease as the mice lacking these cells exhibit decrease in specific IgE, IgG1, and pulmonary IL-5 levels even after the development of pulmonary allergic inflammation. A prominent role for cytolytic T cells ($CD8^+$) in asthmatic disease has also emerged. A series of diverse observations

suggest the involvement of $CD8^+$ cells in allergic disease. These findings include the following:

1. The peripheral blood of individuals with atopic dermatitis has higher numbers of cutaneous lymphocyte-associated antigens $(CLA)^+CD8^+$ T cells, which produce TH2 cytokines.
2. During infancy, wheezing is also associated with the sequestering of $CD8^+$ cells in the airways during an acute asthma attack, and asthma deaths are associated with activated $CD8^+$ T-cell infiltration into peribronchial tissue.
3. The bronchial lavage of atopic subjects' exhibit increased TH2 cytokine (IL-4, IL-5, IL-13) mRNA levels in both $CD4^+$ and $CD8^+$ T cells.
4. A significant infiltration of $CD8^+$ T cells is found in late asthmatic reactions induced by Fel d 1-derived peptides,
5. Allergen-specific $CD8^+$ T cells produce significantly less IL-10 in subjects with severe disease as compared to mild disease. Consequently, $CD8^+$ T cells also play an important role in the pathogenesis of allergic disease.

The Notch ligand Jagged1 (CD339) expressed on DCs is responsible for TH2 differentiation by enhancing the expression of GATA-3 and IL-4 in T cells in vivo. Furthermore, the stimulus for TH2 cell-derived immune responses depends on the expression of the transcription factor IRF4 in DCs. This regulates the maturation of a PD-L2-expressing DC subset 23. IRF4 also induces the expression of IL-33 and IL-10 by DCs, which is essential for the induction of TH2 differentiation. The number of PD-L2+IRF4+ DCs is substantially greater in *Csnk2b*fl/fl*Foxp3*-Cre mice than in *Csnk2b*fl/fl mice, implying that CK2β controls the ability of Treg cells to regulate PD-L2+IRF4+ DCs and TH2 immune responses.

6.11 The Role of Signal Transducers and Activators of Transcription in Allergic Disease

Signal transducers and activators of transcription (STAT) are pivotal in transmitting signals for many cytokines which play an important role in the etiology and pathogenesis of allergic disease. They also impact other cell types including epithelial cells, lymphocytes, mast cells, eosinophils, and dendritic cells by their signaling that has an impact on allergic disease. One role that STATs play in allergic disease is via their effects on regulatory T cells. STAT3 and STAT5 play a role in upregulation of Foxp3 expression by IL-2, and the development of regulatory T cells via IL-2 requires STAT-5-dependent signaling mechanisms.

Allergic inflammation is produced as a result of dysregulation in STAT pathways. Allergen-induced airway inflammation is mediated via STAT6, which also plays an important role in airway hyperresponsiveness, mucus production, chemokine expression, airway eosinophilia, and cell trafficking of TH2 cells. Several genes, including eotaxin-1, eotaxin-3, P-selectin, and arginase 1 are involved in allergic inflammation, and are induced by STAT6. IL-13 and IL-4 are the primary

inducers of STAT6 activation but STAT6-independent pathways are also involved in allergic inflammation. Furthermore, STAT1, STAT3, STAT4, and STAT5 play a role in allergic inflammation, either directly or indirectly. STAT5 is crucial for the activation of mast cells by IgE, and STAT4 is a modulator of airway hyperresponsiveness as well as allergen-induced chemokine production.

6.12 IL-4/Signal Transducers and Activators of Transcription 6 Signaling

IL-4 signaling pathway promotes proliferation and differentiation of TH2 cells, which involves the transcription factor STAT6. After IL-4 binds to IL4Rα, there is dimerization of two receptor subunits, IL4Rα and IL-4Rϒ chains. This leads to Janus kinase-dependent phosphorylation of tyrosine residues present in IL4Rα. There is subsequent binding of STAT6 monomers via their src homology 2 domains to the phosphotyrosine residues located in IL4Rα. Janus kinases produce the phosphorylation. After the dimerization of phosphorylated STAT6 monomers, it translocates into the nucleus. That is where STAT6 regulates the expression of IL-4 genes. After activation of STAT6, it induces the expression of the GATA-binding protein 3 (GATA3), a TH2 master regulator. GATA 3 plays a role in the production of TH2 cytokines. In addition to STAT6; STAT5A and STAT3 also have some role in the development of TH2 cells.

6.13 Notch Signaling Pathway

STAT6-independent mechanisms are also involved in TH2 polarization. One of these pathways is called Notch signaling pathway. There are four Notch proteins, Notch 1, 2, 3, and 4, and they are expressed in the $CD4^+$ T-cell compartments. They are membrane-bound receptors which are heterodimeric and possess an extracellular domain and a transmembrane domain. Jagged family (Jagged 1 and Jagged 2) and delta-like ligands (DLL1, DLL3, and DLL4) serve as ligands for Notch signaling pathway. Notch signals play a role in the expression of IL-4 and GATA3.

6.14 IL-2/Signal Transducers and Activators of Transcription 5 Pathway

IL-2- and IL-2-activated STAT5A cause the expression of IL-4 and therefore are responsible for TH2 priming. The production of IL-4 has been reported even in the absence of IL4Rα and STAT6 by the constitutive induction of STAT5A. Furthermore, changes in STAT5 function had no effect on the expression of GATA3. Naive T cells

can produce IL-4 in conditions favoring TH1 development, if they possess constitutively active STAT5A. Inhibition of IL-2 interferes with the production of IL-4 from TH2 cells, without affecting the GATA3 expression. Increased levels of GATA3 do not affect IL-4 production in STAT5A defective state. This suggests that IL-2/ STAT5A and GATA 3 are not interdependent but can regulate IL-4 production. Using animal models, it has been suggested that STAT5A is essential in STAT6-independent development and differentiation of TH2 cells, as well as TH2 cell-dependent inflammation.

6.15 Mammalian Target of Rapamycin Complex 2 (mTORC2)

Mammalian target of rapamycin (mTOR) plays a role in TH cell's development. There are two types of mTOR complexes, mTOR1 and mTOR2. The mTOR2 complex causes the differentiation of TH2 cells by attenuating the negative feedback inhibitor of cytokine signaling 5. STAT6 induction is inhibited by suppressor of cytokine signaling 5. There is an increase in STAT6 activity as a result of inhibition of suppressor of cytokine signaling 5, which induces TH2 proliferation and differentiation. The disruption of mTORC2 causes the suppression of TH2 development.

6.16 T-Cell Factor 1 (TCF-1)

T-cell factor 1 (TCF1) is a transcription factor present in the nucleus and bound to Wnt response elements. It is involved in the development of TH2 cells. The allergen-induced stimulation of TCR and its activation result in augmentation of the expression of GATA3-Ib via TCF-1 and β catenin. This causes an increase in IL-4 production. Studies in animal models have shown a role of TCF-1 in TH2 polarization, which is independent of STAT6-dependent cascade. TCF-1 also hinders IL-17 production and suppresses the synthesis of interferon ϒ in TH2 cells, which are undergoing development. IL-4 is an inhibitor of short suppressive isoform of TCF-1 in a STAT6-dependent manner.

6.17 Specific Immunotherapy

Clinical tolerance to allergens may be achieved by the administration of allergen extracts which is called specific immunotherapy. In addition to its therapeutic value, it has been suggested that this treatment may also alter the progression of the disease. Specific allergen immunotherapy (SIT) is used to alleviate the condition associated with atopy by administering allergen extracts. Immunotherapy for allergic

disease was first developed at the end of the nineteenth century in England, which was based on the principles described by Noon and Freeman. This is initiated by subcutaneously injecting small but increasing quantities of specific allergens over a period of weeks or months, which is followed by administering maintenance injections every 4–6 weeks for a period of 2–4 years. Two other protocols, referred to as the semi-rush protocol and the rush protocol, have also been used for the treatment of allergic disease by immunotherapy. These are different from the regular up-dosing phase, which are a series of weekly injections. The semi-rush protocol entails the administration of several doses on each day every week, whereas the rush protocol constitutes injections of a series of increasing quantities of allergens in 1 day.

A number of theories have been outlined to explain the mechanism of action of immunotherapy, which includes induction of IgG, inhibition of allergen-specific IgE, inhibition of the recruitment of effector cells, shift from TH2 to TH1 cytokines, and induction of regulatory T cells and T-cell anergy. Originally, it was believed that immunotherapy abolishes atopic responses as a result of inhibiting allergen-specific antibodies. However, during the initial phase of immunotherapy, allergen-specific IgE levels rise, and this is followed by their return to the original levels during the maintenance therapy. It also appears that the systemic beneficial effects of immunotherapy are much more pronounced than the limited observed effects during the skin test. No late-phase skin responses are observed if the patient responds well to therapy. Furthermore, it does not appear that the immunotherapy is beneficial because of the ability of IgG to neutralize the allergens and to inhibit the allergic response since the rise of IgG is observed after the beneficial effects of the therapy, and the allergen first encounters IgE before it is recognized by IgG. The immunotherapy modifies allergen-specific T-cell responses, which include a reduction in eosinophil and T-cell recruitment in response to allergen and increased expression of TH1 cells. The effects on regulatory T cells may be mediated via inducing the ability of allergen-specific B cells to produce IgG4. The effectiveness of immunotherapy in allergic rhinitis and specifically to aeroallergens may last up to 6 years and is more beneficial for seasonal rhinitis than perennial rhinitis. The possible new technologies that are under development for immunotherapy include recombinant allergens, T-cell peptide vaccines, agents which stimulate TH1 cells, allergen–immunostimulant complexes, and hypo-allergenic allergens.

6.18 Sublingual Immunotherapy

Some patients experience discomfort and frequent swelling at the site of the injection. As a consequence, sublingual immunotherapy is used. An extract of allergens is placed under the tongue for a couple of minutes and is then swallowed. The dose for sublingual immunotherapy is 3–300 times greater than subcutaneous immunotherapy. This form of therapy is more effective for seasonal allergens than perennial allergens in both adults and children and its long-term efficacy may be similar to subcutaneous immunotherapy.

6.19 Oralair

Oralair is the first drug (sublingual immunotherapy) to treat multiple grass pollens in the United States. It is indicated for the treatment of patients ages 10–65 years with grass pollen-induced allergic rhinitis. The patients need to be confirmed by positive skin test or in vitro testing for pollen-specific immunoglobulin E antibodies for any of five grass species, sweet vernal, orchard, perennial rye, timothy, and Kentucky blue grass. It is a once-daily tablet that dissolves rapidly under the tongue. The first dose needs to be given in the physician's office. This will allow the monitoring of the patient for any adverse reactions. This immunotherapy is initiated 4 months before and continued throughout the grass pollen season. Oralair should not be given to patients with severe or uncontrolled asthma and history of systemic allergic reactions and/or eosinophilic esophagitis. The side effects include oral pruritus, throat irritation, ear pruritus, tongue pruritus, and cough.

6.20 RAGWITEK

RAGWITEK is a sublingual allergen immunotherapy in the form of tablets for patients suffering from moderate to severe allergic rhinitis to ragweed pollens with or without conjunctivitis. The tablet contains short ragweed pollen allergen extract (Amb a 1-U). This is a treatment for adult patients (18–65 years old) who have declined traditional subcutaneous injections of the allergen. The patients need to be confirmed by positive skin test or in vitro testing for pollen-specific immunoglobulin E antibodies for ragweed. A recommended dose of one tablet daily is placed under the tongue, where it dissolves. The first dose is administered under the supervision of an allergist, and the patient is monitored for at least 30 min after receiving the tablet for any adverse effects. Subsequently, the patient can take the drug at home if no side effects were observed after the first dose but is also provided with auto-injectable epinephrine. Treatment with RAGWITEK begins at least 12 weeks before the predicted onset of ragweed pollen season. RAGWITEK needs to be taken throughout the ragweed pollen season. The treatment with the drug should be stopped if the patient has oral inflammation or wound until the problem is resolved. It is not indicated for relieving the immediate onset of symptoms.

The drug should not be given to patients with severe or uncontrolled asthma and history of systemic allergic reactions and/or eosinophilic esophagitis. The adverse reactions include oral pruritus, throat irritation, tongue pruritus, itching of the lip, mouth edema, and oral paresthesia.

6.21 Food Allergy

Allergies to food have almost reached epidemic levels. About 12 million Americans suffer from food allergies that include almost 1 in 13 children. Allergy to food is a result of an adverse immune response to some protein in the food. Intolerance to

food or food poisoning is not allergic responses. Food allergy is caused by adverse acquired immune response to food proteins. The GI tract processes ingested food for absorption. It neutralizes foreign antigens and blocks them from entering the circulation. The intestine is the largest immune organ in the body, and it protects the body from invading pathogens and ingested proteins. It is achieved by secreting immunoglobulin A (IgA) and activating immune cells which result in the maintenance of homeostasis in the gastrointestinal tract. Gut-associated lymphoid tissue (GALT), specifically Peyer's patches, induces immune response and maintains homeostasis of the gastrointestinal tract. The gastrointestinal epithelium and dendritic cells are crucial in this maintenance by sampling the gastrointestinal luminal contents. A number of interactions between microbes, epithelium, and GALT play a role in the development of the memory of the immune system and as a result cause oral tolerance.

Nonspecific (mucin coat) and specific (secretory IgA) mechanisms "hold up" or block potentially antigenic substances from penetrating the intestinal barrier. Food proteins are the most common allergens, which are recognized by the body as harmful proteins, and as a result, it mounts an immune response. Certain proteins are not completely digested, and these undigested fragments bind to IgE, which are recognized by the body as invading pathogens. There is a migration of leukocytes to the site of the IgE protein complex, resulting in the allergic reaction. The GI symptoms develop within minutes to 2 h of ingesting food. These symptoms include lip and tongue swelling, laryngeal edema, nausea, abdominal cramping, vomiting, and diarrhea. The gastrointestinal symptoms can be present even in the absence of systemic anaphylaxis. They may include both IgE-mediated and T-cell-mediated reactions. There is infiltration of eosinophils in the mucosal and muscular layers of the stomach or small intestine. Basophils play a major role in gastrointestinal reactions. The phenotype of basophils may correlate with the clinical symptoms. There is augmented intestinal permeability, enhanced eosinophilia, and an increase in the number of mast cells. Mast cell-mediated enhancement of epithelial permeability plays a role in oral sensitization.

These responses may also include skin reaction (dermatitis), respiratory and gastrointestinal discomfort, vasodilation, and biphasic anaphylaxis. Atopic patients have higher incidence of developing food allergies. The food allergies could be IgE mediated, IgE and/or non-IgE mediated, and non-IgE mediated. IgE-mediated food allergies are type 1 immediate hypersensitivity reactions that include oral allergy syndrome. IgE- and/or non-IgE-mediated food allergic reactions involve allergic eosinophilic gastritis, allergic eosinophilic gastroenteritis, and allergic eosinophilic esophagitis. Non-IgE-mediated food allergy causes Heiner syndrome, which is a lung disease. This is due to the formation of milk protein/IgG antibody immune complexes in the blood after their absorption through the GI tract. Other examples of non-IgE-mediated food allergy are food protein proctitis/proctocolitis, food protein-induced enteropathy, food protein-induced enterocolitis syndrome, and milk–soy protein intolerance. A common food allergy is a sensitivity to peanuts, and allergies to other nuts including coconuts, pistachios, pecans, pine nuts, and walnuts are also not that uncommon. Some people may be allergic to multiple types of nuts at the same time. Allergic reactions are also produced by eggs, milk,

seeds, seafood (fish, shrimps, prawns, shellfish), wheat, rice, yeast, fruits, and vegetables.

The practice of immunotherapy is also being applied for food allergies. It is administered only under the supervision of an allergist to protect the patient from any adverse effects. These therapies to food allergy include oral immunotherapy and sublingual immunotherapy. In oral immunotherapy the patient is allowed to eat or drink the incremental doses of the food allergen beginning from a low dose. The idea is that such treatment will result in tolerance and desensitization. There are many different protocols for oral immunotherapy. Generally, a low starting dose of the allergen is administered, which is then increased every 2–4 weeks. There is also a buildup phase even before a minimal dose of the allergen is administered. The dose is made up of a protein powder mixed with some type of food such as yogurt or other safe vehicle. Gradually a maximum dose is reached with time and fixed intervals. Using this therapy tolerance is only maintained if desensitized patients are exposed to the allergen regularly. Interestingly, oral immunotherapy in combination with a monoclonal antibody to IgE such as omalizumab or talizumab results in reaching desensitization rather rapidly. In other cases the patients are pretreated with antibody to IgE before immunotherapy, which produces similar results.

In sublingual therapy, allergen is placed under the patient's tongue for absorption. The patients who go through these trials become desensitized to food allergens such as peanuts, egg white, and milk. According to reports desensitization is achieved in 50–75 % of the patients. The sublingual therapy has fewer adverse effects than the oral immunotherapy. However, it is less effective in reaching desensitization and tolerance when compared to the oral immunotherapy. This may be attributed to lower dose levels for sublingual therapy versus oral immunotherapy. The maximum dose level for sublingual therapy is approximately 700 mg, whereas for oral immunotherapy, it is 5,000 mg. The desensitization for both forms of therapy is accompanied by a decrease in IgE and increase in IgG4 levels. Obviously this change is a result of a decrease in the production of TH2 cells and their relevant cytokines. It is feasible that atopic patients may have a deficiency of a specific subset of T cells, which is ameliorated by the desensitization treatment. The treatments culminate with a "discontinuation phase" and a "tolerance food challenge." The last test determines the effectiveness of the immunotherapy. The entire therapy may take 12 months or longer to conclude.

During the oral immunotherapy, adverse effects are common. Other factors such as illness, exercise, and increase in the dose of the allergen exacerbate adverse effects. There is a chance of breakdown in tolerance, after the immunotherapy is completed; therefore continuous exposure to the allergen is required. The endpoint for the treatment of food allergy may not always be tolerance. The treatment may produce desensitization and incremental changes in the antigen dose are required to protect from continued exposure to antigen. However, it is possible that the treatment may result in permanent tolerance. Desensitization may allow protection from the allergic shock as higher doses of allergen could be tolerated.

6.22 Allergy to Seminal Plasma

Allergy to human seminal plasma is a disorder which is most often misdiagnosed. Its exact prevalence is unknown due to the social sensitivity of the problem, although it has been reported worldwide. Human seminal plasma includes the components of human semen with the exception of spermatozoa. Either systemic or local allergic responses are produced by the proteins in human seminal plasma. The systemic reaction to seminal plasma is generally produced within minutes after ejaculation, which includes localized vulvar and vaginal pruritus and/or edema that are followed by nasal obstruction, itching eyes, urticarial and diffuse pruritus, and/or angioedema. There is also involvement of the face, lips, tongue, and throat. Occasionally, respiratory symptoms, wheezing, and GI symptoms are also reported. There has not been any report of death from anaphylactic shock. Local reactions include itching, burning, edema, and vaginal or vulvar pain. The symptoms may begin immediately after exposure or may be delayed up to hours, and both atopic and nonatopic women present this condition. The discomfort in the affected areas may last for several days. The systemic allergic reactions are mostly caused by IgE-mediated reactions. Many allergen proteins are suspect but the available data is rather limited and the results are not conclusive. About 40 % of the effected patients are pre-sensitized to the allergen, suggesting a cross-reactivity and the presence of pre-existing IgE, leading to the manifestation of symptoms. It has been reported that IgE to dog epithelial allergen also binds to human PSA, suggesting cross-reactivity. For diagnosis-specific IgE, assays for seminal plasma are done in vitro which are deemed not to be reliable. Alternatively, skin testing with whole seminal plasma is performed.

Desensitization with immunotherapy is an option. The common procedure utilized is intravaginal desensitization. It is performed with the whole seminal fluid beginning with a lower concentration and gradually increasing the dose at fixed intervals, until an undiluted specimen is deposited. The entire procedure takes about three hours. During the treatment the patient is exposed to the semen two to three times each week, to maintain tolerance. The success rate of this procedure is not very good. Compliance of a post-procedure exposure is also an issue. In some cases a refrigerated sample of semen is administered intravaginally in a clinician's office. After the failure or partial success of this procedure, subcutaneous immunotherapy is performed. The subcutaneous immunotherapy is a 2-day procedure where human seminal plasma fractions are subcutaneously administered beginning with a lower concentration and by gradually increasing the dose. This procedure also requires continuous exposure to the allergen/s, two to three times per week. Based on reports the success rate is improved over the intravaginal desensitization alone, but the short-term/long-term success rate is uncertain. It needs to be pointed out that only a few allergists nationwide perform this potentially therapeutic procedure. Other potential therapeutic options to teat allergic disease in general are depicted in Fig. 6.3.

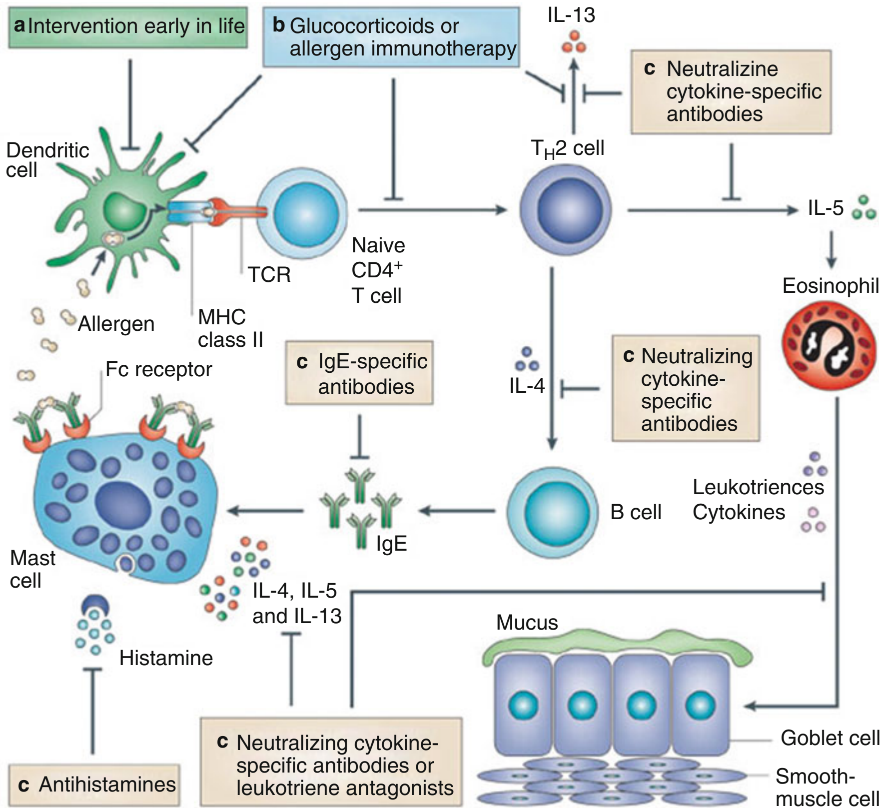

Fig. 6.3 Potential targets to treat allergic disease: Since allergic inflammation is manifested by overproduction of TH2 response, emphasis has been given to modify TH2/TH1 balance either by suppressing TH2 cytokines or augmenting TH1 cytokines. This figure depicts three main sites of treatment. The first option is to intervene early in life and stop overproduction of TH2 responses to allergens. The second avenue is to interfere with the induction of allergen-specific TH2 cells. This could be achieved by directly or indirectly by affecting the antigen-presenting cells by using glucocorticoids or immunotherapy. The last option shown in the figure is to neutralize effector molecules, such as antibodies against TH2 cytokines, IgE, T-cell receptors, and widely used antihistamines and leukotriene antagonists (Source: Reproduced with permission. Hawrylowicz CM, O'Garra A (2005) Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nature Rev Immunol* 5:271–283)

6.23 Gene for Allergic Disease

A protein, Ndfip1, has been identified which protects mice from developing a severe allergic disease that could be fatal. Ndfip1 binds to Nedd4, a family of proteins known as ubiquitin ligases. Ndfip1 blocks the activated T cells to stimulate IL-4 production.

6.24 Ragweed Toll-Like Receptor 9 Agonist Vaccine for Immunotherapy

A novel approach for immunotherapy in allergic disease involves the conjugation of immunostimulatory sequences of DNA to specific allergens. A vaccine which consisted of a ragweed pollen antigen (Amb α1) conjugated to phosphorothioate oligodeoxyribonucleotide immunostimulatory sequence of DNA was tested in subjects with allergic rhinitis to ragweed. This protocol has advantages over the regular immunotherapy methods due to shorter regimens and a low risk of serious adverse effects. In this particular study, the patients were administered six injections once a week of the conjugated allergen and the results demonstrated a substantial reduction in the symptoms of allergic rhinitis. The mechanisms responsible for immune tolerance to the allergen are not fully understood at this time.

6.25 Probiotics for the Treatment of Allergic Disease

Probiotics are the first compounds tested to treat allergic disease which are based on the concept of hygiene hypothesis. The results have shown that the use of probiotics reduces the risk of the development of atopic eczema; however, additional clinical trials are needed before their approval for use in a wider population. Other immunomodulatory compounds derived from bacteria (CpGs), mycobacteria, and helminths are tested to prevent allergic disease.

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Chapter 7
Cellular and Molecular Basis of Asthma

Abstract Allergic asthma is a heterogeneous chronic inflammatory disease caused by a milieu of factors including heredity, allergen exposure, repeated viral infections in childhood, and TH1/TH2 imbalance. Inhalation of common allergens results in atopic sensitization and is characterized by elevated levels of circulating IgE. Allergic sensitization is followed by airway inflammation and remodeling. In this chapter the focus is on the cellular and molecular components of allergic sensitization, airway inflammation, airway hyperresponsiveness, and airway remodeling. This includes TLR, antigen-presenting cells, lymphoid cells, inflammatory cells, chemokines, and a variety of signal transduction factors associated with cytokines and other proteins. A damaged epithelium allows the migration of dendritic cells into the respiratory tract. Dendritic cells which accompany the airway epithelium and underlying mucosa play a crucial role in the uptake of allergens, processing, and presentation to naïve T cells. This pattern creates the environment for the development of TH2 cells and production of IL-4, IL-5, and IL-13. IL-9 is secreted by TH9 cells, and both TH2 and TH9 cytokines lead to the symptoms of asthma. There is detailed explanation of the role played by various cytokines and chemokines in this process followed by inflammatory process resulting in airway remodeling. This is succeeded by a discussion of the role of mitogen-activated protein kinases and the concept of MAPK bistability. Lastly, the future therapeutic options to treat atopic asthma are explored.

Keywords Atopy • Hypersensitivity • Leukotrienes • Prostaglandins • Mast cells • Allergic rhinitis • Allergic asthma • IgE • Eosinophils • Chemotaxis • TCR • HLA • TH2 cells • IL-4 • IL-5 • IL-9 • IL-13 • TH1 cells • STAT6 • GATA3 • Treg cells • Nuclear factor-κB • Dendritic cells • Helper T cells • Cysteinyl leukotrienes • TNF-α • Vascular adhesion molecules • RANTES • TARC • T-bet • Addressin • Integrin • T-cell homing • IL-1 • CCR8 receptors • TLR2 • TLR4 • SPA • IL-8 • IL-1β • Thymic stromal lymphopoietin • CD45RO$^+$, CCR • Foxp3 • Notch-signaling pathway • Immunotherapy • Isotype switching • Mucin • IgA • PAMPs • Damps • Bronchial epithelium • Angiogenesis • Subepithelial fibrosis • Mucus metaplasia • Mass of airway smooth muscle cells • Paucigranulocytic inflammation • FcεRI • Neutrophilic inflammation RORϒt • iNKT • Nuocyte • IL-7 • IL-25 • IL-33 • Goblet cell hyperplasia • CD45RO$^+$ CCR8$^+$ • Mastocytosis • IL-27 • p28 • EBI3 • Type III IFN-λs • IFN-β • IFN-α • CARDIF • TRIF • MyD88 • CCL1–CCL28 • CXCL1–CXCL16 • Eotaxin family • Airway remodeling • Sprouty 2 • Bistability • β-Arrestins

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• ERK1/2 • p38 • Ras • Raf • Mucus metaplasia • MUC5AC • EMTU hypothesis • Neural pathways • Muscarinic M2 autoreceptors • Nonadrenergic–noncholinergic nerves • Tachykinin • Substance P • Neurokinin A • Neurotrophins

7.1 Introduction

Asthma, a heterogeneous disease, is one of the most common inflammatory diseases involving the airways. The prevalence of asthma has been rising worldwide specifically in the industrialized nations over the past 50 years. According to the latest epidemiology study, 235 million people worldwide have asthma, and the death rate was approximately 250,000 individuals per year. The prevalence is higher in the industrialized world but more disadvantaged individuals are affected; however, in the developing countries, more affluent population has the disease. The reason behind this discrepancy is not known. It has been suggested that this is due to urbanization and changes in diet and lifestyle. The urban–rural divide is exhibited in children who are raised with microbes' exposure, are growing up on a farm or with a more natural diet and lifestyle, and have lower incidence of atopic asthma in correlation with their exposure to microbes. The microbial exposure during the mother's pregnancy is even more protective in developing an atopic state. The urbanization has resulted in the loss of microbial biodiversity in the environment and food, and the use of modern farming methods, disinfectants, and antimicrobials in rural areas along with change in the diet has contributed to the increased numbers in developing atopy and asthma in the Western nations. In contrast, the protective immunity is rendered by the amount and levels of exposure to microbes instead of coming in contact with a given organism. Severe asthma occurs at equal rates in boys and girls though it is twice as common in boys as girls. However, among adults the prevalence of asthma is higher in women than men. In industrialized countries 40 % of children and adults develop atopy but only one third of them are asthmatic. New asthma susceptibility genes have been identified that include *ORMDL3*, *IL33*, and *SMAD3* and are predominantly expressed in the innate immune pathways and epithelium. It is possible that the levels of triggering of innate immunity determine the outcome for the development of atopy. Low doses of exposure to PAMP (pathogen-associated molecular pattern) and DAMP (damage-associated molecular pattern) molecules in the presence of allergen will induce allergic response due to priming of dendritic cells, whereas high-dose exposure induces allergenic tolerance. The disease generally begins in early childhood with the symptoms of intermittent wheezing and shortness of breath. The clinical symptoms of asthma result from the narrowed airways due to inflammation. Inflammation in asthma mostly involves the large airways, but in more severe cases, smaller airways and the lung parenchyma are also involved. Persistent inflammation of the airways results in the structural changes in the lung that affect airway smooth muscles, epithelial lining, extracellular matrix, fibroblast layer, and endothelial lining of the bronchial walls. The classic characteristics of asthma include induction and recruitment of cells involved in inflammation, shedding of bronchial epithelium,

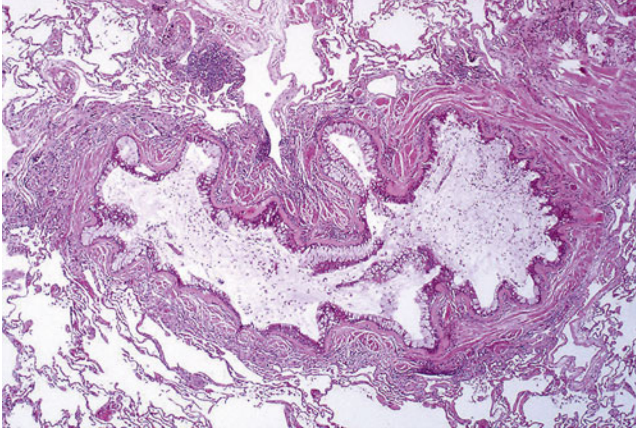

Fig. 7.1 Pathology of airways in asthma: This figure depicts the obstruction of the lumen of the bronchioles by mucus production, metaplasia of goblet cells, thickening of the epithelial basement membrane, and inflammation of bronchiole (Source: Yale Rosen. Creative Commons-Attribution-Share Alike 2.0 generic)

angiogenesis, subepithelial fibrosis, mucus metaplasia, and changes in the mass of airway smooth muscle cells (Fig. 7.1).

The physical barrier function of epithelium in asthmatics is innately defective as it shows incomplete formation of tight junctions. This allows entry of allergens into the airways. Some allergens themselves are capable of entering the epithelial barrier and transmit a signal to dendritic cells. A few examples of allergens, which are proteolytic and can alter epithelial tight junctions, include house dust mite, animal allergens, fungal allergens, and cockroach. In addition, viral infections, tobacco smoke, and other air pollutants disrupt epithelial barrier function through disruption of tight junctions.

The development of allergic asthma can be divided into two stages: an allergic sensitization phase and a second phase of the development of the disease, which is characterized by airway inflammation and remodeling. In atopic patients allergic sensitization takes place after subsequent exposure to the allergens and their presentation to T cells in the presence of dendritic cells in context of MHC class II molecules. This results in the differentiation of naïve CD4+ T cells into TH2 cells. IL-4 is crucial in TH2 differentiation. Activation of IL-4 receptors results in the activation of TH2 lineage-specific transcription factor GATA3, which binds to target regulatory sequence of IL-4, IL-5, IL-9, and IL-13, which are all TH2 cytokines and play roles in allergic disease and asthma.

7.2 Role of Viral Infections in the Development of Asthma

Rhinovirus infection and its induced wheezing in early childhood in individuals genetically predisposed to the disease are a risk factor for asthma. A repeated rhinovirus exposure at 3 years of age leads to an increased risk of the development of

asthma at age 6. Both upper and lower airway epithelial cells are the main site of rhinovirus infection. The viral infection also affects the degree of allergen sensitization. The clinical outcome is dependent on alterations in epithelial cells. This etiology may reside in the inability of the airway epithelial cells to produce normal levels of interferon-β and IFN-ϒ in response to TLR3. A lack of antiviral cytokine production results in repeated viral lung infection and as a consequence allergic sensitization. Under these circumstances there is viral replication, increased viral shedding, release of pro-inflammatory mediators (cytokines, chemokines, and growth factors), and cytotoxic cell death. The susceptibility of asthmatic patients may reside in the relative induction of epithelial host innate antiviral responses versus pro-inflammatory responses. Therefore, there is a damage to epithelial lining, recruitment of immature dendritic cells, and their priming for allergen sensitization. The patients with asthma in response to rhinovirus infection produce TSLP from epithelial cells, which is a pro-TH2 cytokine, and it's effects are antagonized by IFN-β.

7.3 Cellular Component

Asthma is characterized by airway inflammation, airway hyperresponsiveness, and airway remodeling. The inflammatory and other responses result from the infiltration of eosinophils, neutrophils, mast cells, and monocytes/macrophages. In addition, many inflammatory proteins that include cytokines, chemokines, adhesion molecules, and their receptors when responding to their respective agonists contribute to the disease. There are at least three pathological phenotypes of asthma based on the involvement of the subtype of the leukocyte; eosinophilic, neutrophilic, and paucigranulocytic. Nonetheless, asthma is a heterogeneous disease with a range of yet undefined phenotypes. There is an understanding that inflammation in asthma is induced by a subset of TH2 cells, which are responsible for eosinophilic allergic inflammation. Asthma is a mixed profile contributed by TH1, TH2, TH9, and TH17 cells. TH1 and TH17 cells are responsible for neutrophilic inflammation in the lungs of some patients with severe asthma who are refractory to glucocorticoid treatment. Neutrophilic inflammation may also be involved in acute and viral episodes of asthma. A large number of neutrophils in the airways of patients have been found in patients who die suddenly. The high doses of corticosteroids promote neutrophil survival and suppress their apoptosis. The infiltration of eosinophils, TH2 cells, and activated mast cells has been observed in bronchial biopsies of asthmatic patients.

Dendritic cells are a special class of antigen-presenting cells, which accompany airway epithelium and underlying mucosa. They exhibit receptors of the innate immune system and are also capable of allergen uptake, processing, and presentation to naïve helper T cells in context of MHC molecules. The uptake of allergens by airway dendritic cells is supported by allergen-specific IgE bound to FcεRI. At birth, the dendritic cells are not present in the airways. The influx of immature dendritic cells into the respiratory tract results from exposure and damage of the respi-

ratory epithelium by infection and/or other irritants. This leads to the release of chemokines and migration of dendritic cells to the site of epithelial injury. The dendritic cells reach full maturation in response to various cytokines (GM-CSF, IL-4, TNF-α), and there is a shift in TH1/TH2 paradigm favoring the development of TH2 cells, in response to allergen recognition on dendritic cells. Biological and proteolytic characteristics of allergens produce signals (PAMPs) for the maturation of dendritic cells. Pattern recognition receptors (PRRs) are involved in allergen sensitization. After contact with allergen, induction of pattern recognition receptors increases their homing capacity. This is achieved by upregulation of chemokine receptors. The myeloid dendritic cells are involved in antigen presentation. All microbes possess small amounts of TLR ligands, which, when inhaled in the presence of allergen, drive the allergic response by being adjuvant in a nature. The motility of dendritic cells is enhanced by activation of selective TLRs on epithelial cells. This is the result of the secretion of chemokines (CCL17 and CCL22 via CCR4), which support TH2 cells and cytokines (IL-25, IL-33, GM-CSF, and thymic stromal lymphopoietin [TSLP]). IL-4, STAT6, and GATA3 play an essential role in this process. The genes associated with atopy include FCER1A, STAT6, and IL-13.

The explanation of asthma as an eosinophilic airway disease is described by the imbalance of TH1/TH2 cytokine paradigm. The upregulation of GATA3 and downregulation of T-bet (TH1-inducing transcription factor) are observed in T cells obtained from the airways and peripheral blood of patients with asthma. GATA3 and T-bet also play roles in airway remodeling by affecting TH1/TH2 differentiation. The overexpression of GATA3 results in elevation of allergen-specific IgE levels; enhanced expression of IL-4, IL-5, and IL-13 by the lungs, eosinophilic airway inflammation, mucus hypersecretion, and goblet cell hyperplasia, whereas; overexpression of RORϒt predominantly caused the infiltration of neutrophils into the airways, and the expression of IL-17A and IL-22 is enhanced in the lungs. There is an increase in the levels of IL-17 in the airways of persistent asthmatic patients. The exact role of RORϒt in asthma has not been elucidated, but according to reports there is increased expression of RORϒt or RORC, which encodes for RORϒt, in asthmatics. As a consequence, increased levels of RORϒt may play a role in the mechanisms responsible for asthma.

The patients with allergic asthma exhibit a decrease in the number of Treg cells in airways. This results in enhanced effector T-cell proliferation and cytokine production when compared with individuals without atopic asthma. It has been reported that successful corticosteroid treatment results in enhanced expression of $Foxp3^+$ Treg in asthmatic patients and an induction in their effector function.

7.4 Role of Invariant Natural Killer T Cells

The invariant natural killer T (iNKT) cells recognize the glycolipid α-galactosylceramide (α-GalCer) during the presentation to the TCR in context of antigen-presenting molecule, CD1d. This cell type plays a role in asthma. iNKT

cells are involved in airway hyperresponsiveness, which is either in combination with TH2 cells or independent of acquired immune response. It is likely that these cells modulate the asthmatic phenotype instead of being the critical drivers. Some antigens in the house dust extracts induce human iNKT cells.

7.5 Role of Nuocytes

The identification of innate type II immune effector leukocyte, the nuocyte, provides a missing link between the innate and acquired TH2 responses. IL-7 and epithelial derived IL-25 and IL-33 cause the proliferation of nuocytes. Nuocytes recruit other cell types through the production of IL-4, IL-5, and IL-13. They are a novel population of TH2 cytokines producing cells. Therefore, nuocyte plays a role in allergic airway disease, in addition to expulsion of helminth worms. These cells have a lymphoid origin and their developmental pathway is dependent on RORα and Notch signaling. They arise during airway lung inflammation and contribute to airway hyperresponsiveness.

7.6 Role of Cytokines

The chronic inflammation in asthma is the product of the actions of cytokines. These cytokines play a crucial role in the recruitment, induction, and differentiation of various types of inflammatory cells in the respiratory system. While the precise role of most of the cytokines in the etiology and pathogenesis remains to be clearly delineated, more than four dozen cytokines have shown some role in the disease. T cells play a crucial role in asthma by releasing a number of cytokines, which recruit various inflammatory cells, in addition to regulating structural cells in the airways. Specifically, there is an increase in the number of TH2 cell cytokines in asthmatic patients, which secrete IL-4, IL-5, and IL-13. IL-9 which is secreted by TH9 cells also plays a role in the disease (Fig. 7.2). These cytokines are relevant to asthma, as TH2 cells also secrete additional cytokines. The differentiation of uncommitted naïve T cells to TH2 cells requires GATA3, which is a transcription factor. Asthmatic patients exhibit increased number of GATA3+ T cells in the airways (Fig. 7.3). After allergen recognition, GATA3 is phosphorylated and activated by p38 MAPK, which results in its translocation from the cytoplasm to the nucleus. In the nucleus activated GATA3 induces TH2 cell-specific gene characteristics. The transcriptional activation of GATA3-induced IL-4 promoter is augmented by nuclear factor of activated T cells (NFAT). Asthmatic patients have a decrease in the expression of T-bet, which is an inhibitor of GATA3, thus resulting in lower expression of TH2 cells (Fig. 7.4). TH1 cells predominantly secrete IFN-ϒ, and its levels are low in asthmatic individuals. Individuals with severe asthma during the asthma attack have elevated levels of IFN-ϒ. STAT1 mediates IFN-ϒ-dependent activation of T-bet.

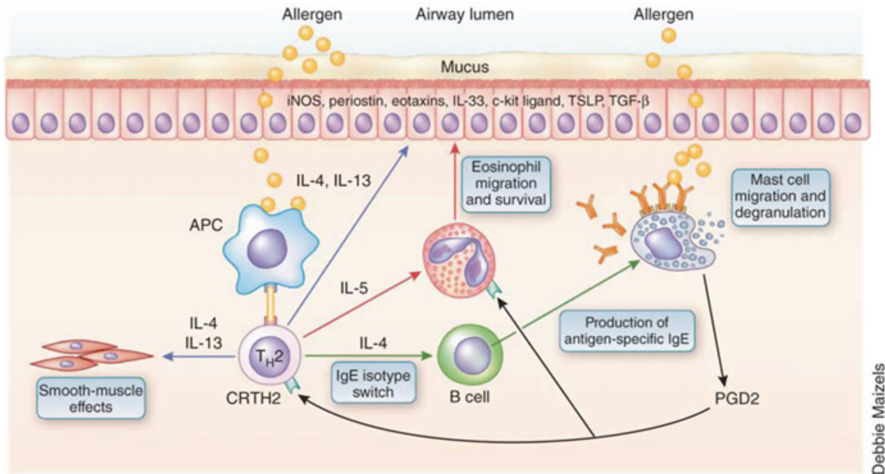

Fig. 7.2 TH2 immune responses in asthmatics: The development of TH2 cells from naïve T cells in response to allergens results in production of TH2 cytokines, IL-4, IL-5, and IL- 13. These cytokines play a crucial role in the induction of allergic and eosinophilic inflammation. Furthermore, they cause epithelial and smooth muscle modification which results in the pathophysiology of asthma. There is activation of antigen-presenting cells and increased expression of chemoattractant receptor homologous molecules present on TH2 cells, prostaglandin D2, induced nitric oxide synthase, and thymic stromal lymphopoietin (Reproduced with permission. Source: Wenzel SE (2012) Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature Med* 18:716–725. Nature Publishing Group)

IL-4 is essential in the development of allergic disease and asthma. The asthmatic patients have increased levels of IL-4 in their serum and bronchoalveolar lavage fluids, increased levels of IL-4 mRNA, and higher levels of IL-4 mRNA expressing T cells in bronchial biopsies as well as bronchoalveolar fluid. There is also enhanced expression of IL-4 receptors in bronchial biopsies of asthmatic patients. Furthermore, there is an association between atopic asthma and genetic polymorphism in the IL-4 gene and IL-4 receptor gene. The differentiation of TH2 cells from their precursors is mediated by IL-4, which forms the basis of the synthesis of IgE in response to an allergen instead of IgG.

IL-13 is an important cytokine in asthma and affects airway hyperresponsiveness, mucus production, airway remodeling, and subepithelial airway fibrosis. It is involved in many of the pathologic events in different asthma phenotypes. A number of regulatory signals for activation and inactivation are associated with IL-13. It does not promote TH2 differentiation, but is involved in isotype switching and producing changes in the structure of the airways. Goblet cell hyperplasia and mucus overproduction are characteristics of asthma and cause airway blockage which is responsible for death in asthmatic patients. In human in vitro studies, IL-13 produces goblet cell hyperplasia and MUC5AC expression and enhances bronchial epithelial periodic acid–Schiff (PAS) cell staining. The severity of asthma is correlated with human epithelial 15-LO1 expression. IL-13 activates 15-LO1, which

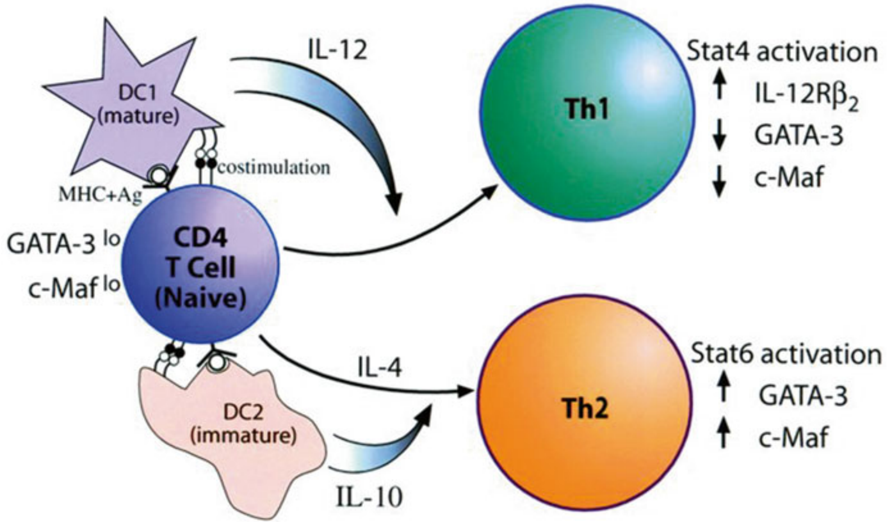

Fig. 7.3 Differentiation of TH1 and TH2 cells and the levels of GATA3 in allergic inflammation/asthma: The mature dendritic cells secrete IL-12 and cause TH1 differentiation, whereas immature dendritic cells secrete IL-10 and cause TH2 differentiation. The cytokines (IL-4/IL-10 or IL-12) present in the microenvironment are also responsible for this differentiation. Differentiation of uncommitted naïve T cells to TH2 cells requires GATA3, which is a transcription factor. The transcriptional activation of GATA3 induces IL-4 promoter (Reproduced with permission. Source: Ray A, Cohn L (1999) TH2 cells and GATA3 in asthma: New insight into the regulation of airway inflammation. *J Clin Inv* 104:985–993. Copyright: American Society for Clinical Investigation)

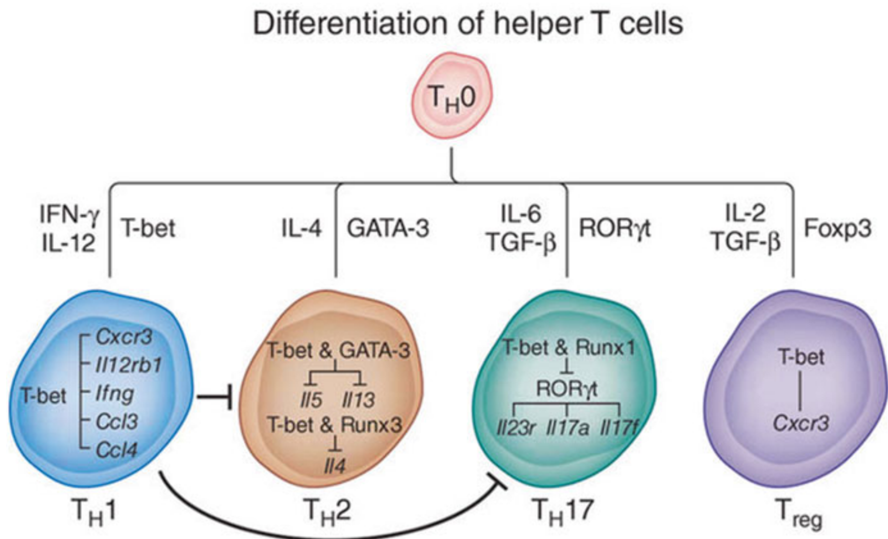

Fig. 7.4 Role of T-bet in asthma through its effects on cytokines (Reproduced with permission. Source: Lazarevic V, Glimcher LH (2012) T-bet in disease. *Nature Immunol* 12:597–606. Nature Publishing Group)

stimulates formation of 15-HETE that is metabolized through esterification to produce phosphatidylethanolamine (15-HETE-PE). Induction of 15-HETE-PE by IL-13 augments MUC5AC expression in human airway epithelial cells.

The common effects of IL-4 and IL-13 are mediated through activation of STAT6. The structural changes induced by IL-13 in chronic asthma include proliferation of airway smooth muscles, subepithelial fibrosis, and goblet cell hyperplasia. IL-13 causes inflammation in asthma via several chemokines as well. The airways of asthmatic patients have increased levels of IL-13. Due to diversity of roles played by IL-13 in asthma, it has been a major focus for the development of therapeutic agents, which target this cytokine to treat asthma.

IL-5 is responsible for the differentiation and survival of eosinophils. These cells play an important role in asthmatic inflammation. However, several studies have shown that a monoclonal antibody directed against IL-5 does not ameliorate the disease. According to one explanation, the antibody was not able to remove all the eosinophils in the airways, and as a result it affected the therapeutic benefits of the antibody. Since asthma has different phenotypes, pharmacologic inhibition using antagonists of IL-4, IL-5, and IL-13 will only be beneficial in a specific subset of patients. From this perspective, IL-4 and IL-13 need further investigation in order for their antagonists to be useful tools for treating asthma.

IL-9 is secreted by allergen-specific T cells, human memory ($CD45RO^+$ $CCR8^+$) T cells, iT regulatory cells in peripheral tissues, basophils, mast cells, and eosinophils, depending on the stimulus. It plays a detrimental role in the pathogenesis of asthma. IL-9 increases synthesis and secretion of other cytokines and IgE. There is enhanced mucus hyperplasia, airway hyperresponsiveness, and mastocytosis and proliferation of B cells as a result of increased expression of IL-9. In the airways of asthmatic patients, the expression of IL-9 and its receptors is augmented. It has been shown in experimental models that antagonism of IL-9 suppresses pulmonary eosinophilia, mucus secretion, and airway hyperresponsiveness. In mouse models the effects of IL-9 on pulmonary eosinophilia, mucus secretion, and airway hyperresponsiveness are mediated via IL-13. Genetic studies on a mouse model of asthma have shown that IL-9 is a determining factor in the pathogenesis of bronchial hyperresponsiveness.

The major cell source of IL-9 is TH9 cells, a subset of helper T cells. Their differentiation requires the balanced presence of a combination of IL-4 and TGF-β. As a consequence, both TH2 and TH9 cells share the requirement for transcription factors including STAT6, GATA3, and IRF4. These cells do not express transcription factors such as T-bet, GATA3, RORγt, and Foxp3. PU.1 is an ETS family transcription factor, which induces the development of TH9 cells. It suppresses the development of TH2 cells that makes it a switch factor between the two subsets. In addition, the expression of TH9-enriched genes requires a transcriptional regulatory network. The activator protein 1 (AP1) family transcription factor BATF (B-cell, activating transcription factor-like) is included in the genes present in TH9 cells and is required for the expression of IL-9 and other TH9 genes in human T cells. It has been reported that TH9 cells lead to allergic inflammation in the lung. These cells play significant role in the onset and progression of allergic disease, specifically asthma, via release of large amounts of IL-9.

The levels of IL-12 are also lower in individuals with asthma. The expression of IL-12 receptors is inhibited by IL-4, resulting in the suppression of TH1 cell differentiation. IL-12 is also associated with a decrease in circulating eosinophils. It is produced by macrophages, dendritic cells, and epithelial cells and is important in the differentiation and effector function of TH1 cells. IL-4 is a suppressor of the expression of IL-12 receptors and as a result is an antagonist for the differentiation of TH1 cells. IL-12 is a stimulus for the production of IFN-ϒ from activated T cells. IFN-ϒ regulates the expression of IL-12 receptors and is responsible for the differentiation of TH1 cells. There is a decrease in the secretion of IL-12 by asthmatic patients. The majority of the effects of IL-12 are mediated via STAT4.

IL-27 has both pro- and anti-inflammatory effects in asthmatic disease. The heterodimeric cytokine IL-27 (composed of p28 and EBI3) has a role in limiting TH1, TH2, and TH17 responses, and it can promote T-cell production of IL-10. It has the ability to inhibit inflammation and airway hyperreactivity in mouse asthma models. In humans, its effects on T cells are complicated and are dependent on timing, signaling pathways, and the surrounding microenvironment. The responses to IL-27 can be altered by modulating SOCS3–STAT1 axis, resulting in an anti-inflammatory effect in asthmatic disease. Furthermore, the differentiated TH2 cells can resist IL-27-induced reprogramming toward TH1 cells by downregulating STAT1 phosphorylation. This may explain the reason behind the resistance to IL-27-mediated inhibition in $CD4^+$ T cells of asthmatic patients.

Increased viral exacerbations are seen in asthmatic patients due to decreased expression of antiviral cytokine in epithelial cells. After rhinovirus infection, bronchial epithelial cells (BECs) respond by secreting innate interferon (IFN), which includes type I IFN-β and type III IFN-λs. IFN-λs are antiviral cytokines, which act on their own separate receptors, but have features that are similar to type I IFNs IFN-β and IFN-α.

The rhinovirus-induced production of type I interferon is delayed and deficient in bronchoalveolar lavage cells in asthmatic individuals. The lower interferon levels are associated with increased airway hyperresponsiveness. Some have reported a lack of effect on the expression of Toll-like receptor (TLR) 3, TLR7, TLR8, melanoma differentiation-associated gene 5 (MDA-5), retinoic acid-inducible gene I (RIG-I), and TIR domain-containing adapter-inducing IFN-β (TRIF). Others included in the group are caspase recruitment domain adapter-inducing IFN-β (CARDIF), IL-1 receptor-associated kinase 4 (IRAK4), myeloid differentiation primary response gene 88 (MyD88), interferon regulatory factors 3 and 7, and rhinovirus-mediated expression of the virus-inducible molecules IκB kinase-β (IKKB) and IκB kinase I (IKKI).

IFN-β, IFN-λ1, and IFN-λ2/3 mRNA levels are about 600 times lower in bronchial epithelial cells obtained from severe therapy-resistant asthma children. The expression of TLR3 is also lower in bronchial epithelial cells from severe therapy-resistant asthma children. Furthermore, after 24 h of infection, there is an in inhibition of the virus-induced RIG-like helicases (RLHs), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA-5) mRNA in bron-

chial epithelial cells from severe therapy-resistant asthma children. There is an elevated level of rhinovirus replication, but levels of CXCL8/IL-8 and CXCL5/ENA-78 cytokines and their mRNA are normal. Interestingly, the levels of IFN-ϒ in the airway wall are higher in severe than moderate asthma. IFN-γ is a mediator of TH1 immune responses, whereas TH2 responses dependent on IL-4 and IL-13 are involved in airway inflammation. The TH2-mediated effects are not required in severe asthma, but there is involvement of IFN-ϒ. This suggests a mixed contribution of TH1, TH2, TH9, and TH17 cells in asthma.

IL-17 has a putative role in acute severe and chronic asthma and its levels are increased in the airways of asthmatics. TH17 cells play a crucial role in orchestrating allergic airway inflammation by increasing neutrophil recruitment to the lung and are also involved in corticosteroid insensitivity in asthmatic patients. Chronic TH17 inflammation results in airway remodeling and responses to allergen and increased neutrophilia. There is an increase of IL-17 levels in the circulation and airway fluids in asthmatic patients. IL-17 and its upstream mediator, IL-23, can augment or attenuate TH2-mediated inflammation, and these effects are time and cell source dependent. RORϒt, a member of the nuclear receptor superfamily, is a regulator for TH17 differentiation in the presence of TGF-β and IL-6. TH17 cells are directly involved in neutrophilic airway inflammation. The production of CXCL8, a neutrophil chemokine, which is induced in airway secretions of asthmatics is associated with TH17 cells.

IL-1α and IL-1β are pro-inflammatory cytokines, which act via IL-1 receptor type 1 (IL-1R1). Studies in a mild asthma model suggest that in IL-1R1-deficient mice, the pulmonary eosinophilic inflammation and goblet cell hyperplasia are significantly reduced. Furthermore, priming of $CD4^+$ T cells in bronchial lymph nodes and their recruitment to the lung is altered in IL-1R1-deficient mice. In a severe asthma model, IL-1 plays an important role in pulmonary allergic TH2 responses, in reference to eosinophilic inflammation, antibody responses, and $CD4^+$ T-cell priming in lymph nodes. There appears to be a critical role of IL-1/IL-1R1 in the development of allergic TH2 responses.

7.7 Role of Chemokines

Chemokines play an important role in the accumulation of inflammatory cells. Their ligands comprise of two large subfamilies; CCL1 through CCL28 and CXCL1 through CXCL16. The two small subfamilies include XCL1 through XCL2 and CXCL16. The chemokine receptors are present on TH1, TH2, TH9, and TH17 cells. CCR5 and CXCR3 are expressed primarily on TH1 cells; CCR3, CCR4, and CCR8 are expressed on TH2 cells; CCR3, CCR6, and CXCR3 are present on TH9 cells; and CCR6 is expressed on TH17 cells.

The chemokine and their receptor systems play important roles in asthma. The production of chemokines is related to the severity of asthmatic inflammation.

Significant levels of chemokines are produced by epithelial cells and macrophages. Eotaxin preferentially recruits eosinophils and binds to a single receptor CCR3, which is expressed in large numbers on eosinophils. It is produced in high levels in the airway epithelium in asthmatics and regulates eosinophil's migratory functions. This results in preferential targeting of the epithelium by eosinophils due to the release of the epithelium-damaging proteins. Furthermore, eotaxin degranulates basophils and is a chemoattractant for TH2 cells. A number of chemokines including CCL5 (RANTES), CCL7 (monocyte chemotactic protein-3 [MCP-3]), CCL11 (eotaxin-1), and CCL13 (MCP-4) are responsible for the induction of eosinophil recruitment, activation, and degranulation. These chemokines act via CCR3 receptors. The eotaxin families of chemokines including CCL11, CCL24 (eotaxin-2), and CCL26 (eotaxin-3) bind to CCR3, and all possess profound effect on the movement of eosinophils. Furthermore, a number of non-CCR3-binding chemokines including CCL-3 (MIP-1a) and CCL22 (MDC) also regulate the function of the eosinophils. The neutrophil-activating CXC chemokines are important in airway remodeling as the presence of neutrophils plays an important role in this process. This is attributed to angiogenic property of a subgroup of neutrophil-activating CXC chemokines. In addition, eosinophils exhibit increased expression of CXCL5 (epithelial cell-derived neutrophil-activating peptide-78) that explains the ability of eosinophils to recruit and activate CXCR2-expressing cells, which include neutrophils.

Augmented local secretion of the chemokines CCL1 (I-309), CCL11 (eotaxin-1), CCL13 (MCP-4), CCL17 (TARC), and CCL22 (MDC) is caused by TH2 cytokines including IL-4, IL-5, IL-9, and IL-13. Based on the expression of chemokine receptors, CCR4 (ligands CCL22, CCL17) and CCR8 (ligand CCL1), their local role in TH2-mediated effects seems crucial. Furthermore, repeated allergen exposure results in the utilization of CCR4 pathway, as opposed to the predominant use of CCR3, which is the case in early stages of atopic asthma. There is an association between allergic airway inflammation and high levels of CXCL9 (MIG) and CXCL10 (IP-10). Both of these chemokines bind to CXCR3. CXCL9 inhibits eosinophils, if CCR3 is interfered. CXCL9 binds to CXCR3, which is expressed predominantly on TH1 cells, whereas CCR3 is preferentially expressed on TH2 cells. The inhibition of eosinophils by CXCL9 (MIG) may have clinical implications. Transforming growth factor-β and IL-13 work in synergism to result in the expression of CCL11 (eotaxin-1), by airway fibroblasts. Structural cells, which contribute to airway remodeling, secrete chemokines and express their receptors. The severe asthmatics have high levels of CCL11 (eotaxin-1) – positive fibroblasts in their biopsy samples. CX3CL1 (a type I transmembrane protein with the CX3C chemokine domain) worsens in allergic disease and plays a major role in the development of asthma. However, unlike the other chemokines, it does not direct the migration of immune cells into the airways. Instead, it promotes the survival of TH2 cells in the inflamed airways. This highlights an important distinct function for CX3CL1 and CX3CR1 in atopic asthma.

7.8 Role of Toll-Like Receptors

Toll-like receptors (TLRs) are a class of proteins that play a key role in innate immunity. They play an important role in allergic airway inflammation via activation of the cells of the innate immune system. TLR ligands directly induce the secretion of pro-inflammatory cytokines and chemokines from mast cells without the degranulation of endogenous mediators. They are also expressed on immature pulmonary dendritic cells. Infection and inflammation result in the activation of mast cells and dendritic cells and upregulation of co-stimulatory molecules. Dendritic cells primed by TLR present an allergen to naïve helper T cells resulting in their proliferation to TH2 cells, which release IL-4, IL-5, IL-9, and IL-13. TH2 cytokines affect the proliferation and infiltration of eosinophils and increase the mucus production by goblet cells. These events will result in the chronic airway inflammation of the airways. TLR4 expression is required for the activation of dendritic cells and the induction of T cells in the asthmatic lung, and its antagonists produce a decrease in eosinophilia and lymphocytosis, inhibition of IL-5, IL-13, and IFN-γ production, lower goblet cell hyperplasia, and suppression of airway hyperresponsiveness. The effect of LPS, a TLR4 agonist, on exacerbation of asthma is dose dependent. Low doses of LPS in combination with the allergen increase neutrophil- and eosinophil-mediated lung inflammation. Furthermore, there are high concentrations of TH2 cytokines IL-4, IL-5, and IL-13 and mucus hypersecretion, in response to LPS. Higher doses of LPS induce TH1 response with increased production of IFN-ϒ and recruitment of neutrophils to the lungs. There is also rising evidence for a functional role of TLR3 in the pathogenesis of severe asthma. TLR3 ligands, in combination with thymic stromal lymphopoietin, cause the activation of human dendritic cells and as a result produce the differentiation of TH17 cells. TH17 cells produce IL-17 and IL-25, which play a role in the pathogenesis of asthma. It is possible that the effects of TLR3 activation in producing asthma are mediated via TH17 cell (Fig. 7.5).

TLR2 has immune-regulating activities and is of interest in atopic asthma. TLR2 activation suppresses TH2 responses which are induced by mite allergens. In experimental animal models, TLR2/4 agonist causes the inhibition of airway inflammation, a decrease in the production of TH2 cytokines, and eosinophilia. Furthermore, TLR2/1 and TLR2/6 heterodimers alter TH1/TH2 balance, resulting in the production of more TH1 cells. Another TLR2/6 agonist in combination with IFN-ϒ causes inhibition of airway hyperresponsiveness, production of asthma-relevant TH2 cytokines, and eosinophilia. The data regarding the effects of TLR2/1 agonists in animal models of allergic asthma is conflicting. In one study TLR2/1 agonists reversed OVA-induced asthmatic inflammation, while in another study there was induction in TH2 responses and worsening of symptoms. Both studies were conducted in a similar animal model. Based on several studies, it appears that TLR2 homo- and heterodimer agonists are able to both suppress and induce allergic inflammation. These effects are TLR2 heterodimer specific. There are reports that TLR7–TLR8 ligand protects against airway remodeling in an animal asthma model. It has been found

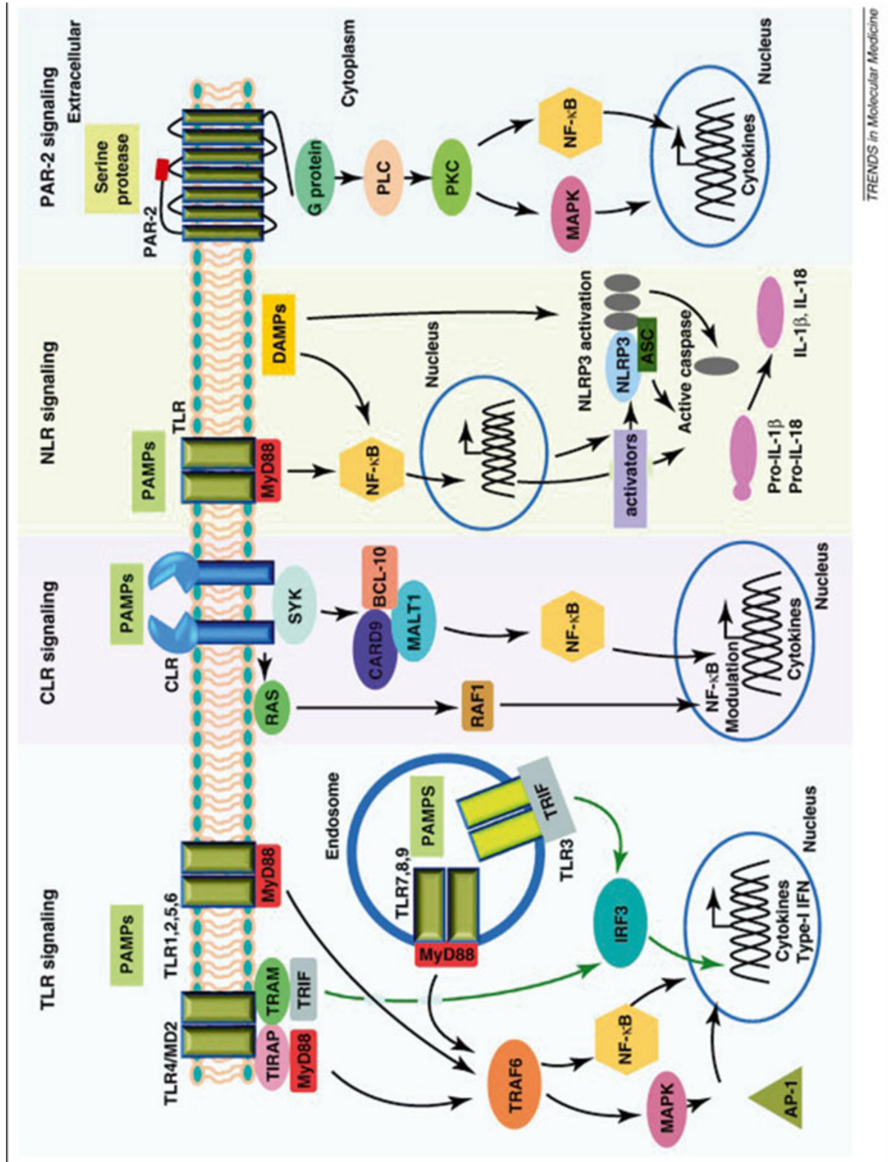

Fig. 7.5 Role of Toll-like receptors in asthma: The activation of TLR results in the recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MyD88) or TIR domain-containing adapter-inducing interferon-β (TRIF). The recruitment of MyD88 causes activation of TNF receptor-associated factor 6 (TRAF6) which results in translocation of nuclear factor-kappa B (NF-κB) to the nucleus. Furthermore, there is also activation of MAPK cascade causing the induction of AP1. Both transcription factors are responsible for the induction of several cytokine genes. Pro-inflammatory cytokine genes and type I interferons are induced by interferon regulatory factor 3 (IRF3), and NF-κB is activated by TRIF-dependent signaling. Other receptors and pathways {NOD-like receptors and proteinase-activated receptor (PAR) signaling pathways} in the induction, processing, and/or activation of NF-κB, IL-1β, and IL-18 (Reproduced with permission. Source: Jacquet A (2012) The role of innate immunity activation in house dust mite allergy. *Trends Mol Med* 17:604–611. Cell Press)

that the ligand suppressed increase in airway smooth muscle mass and goblet cell hyperplasia. There was reduction in the expression of both TH1 and TH2 cytokines in the lungs. In another study induction of TLR7 induced airway hyperresponsiveness in differing circumstances.

Allergens and viral infection play a dominant role in acute asthma attacks. In addition, exposure to air pollution may also be a contributing factor. A role for TLR has been suggested in protecting atopic individuals from acute asthma attacks. If TLR produced a weak response, it could result in the worsening of the symptoms. This is attributed to poor recognition of bacterial or viral antigens by TLR, accompanied by a compromised function of phagocytosis by bacteria and cell death. TLR may also overreact, which will produce enhanced inflammatory response and as a result damage of the lung tissue.

A number of synthetic inducers of TLR are being clinically evaluated to treat asthma. They include agonists for TLR3, TLR4, TLR5, TLR7, and TLR9. Since inflammation plays a key role in the symptoms of asthma, the TLR agonists will be a useful tool for viral- or bacterial-induced acute asthma attacks. This therapy would be helpful in saving the tissue from destruction as a result of inflammation. Furthermore, when the infection is eliminated, TLR antagonists may be employed to restore homeostasis. Several studies have been done in asthma models using TLR ligands CpG ODNs in both preclinical and clinical settings. These ligands enhance the production of several cytokines including IL-12, IL-6, and IFN-ϒ from CD4+ T cells, NK cells, and B cells. Collectively, there is induction of TH1 inflammatory response. There is production of Tregs (regulatory T cells) after the treatment of dendritic cells with CpG ODN, which is mediated by IL-10 and IL-12. CpG ODNs suppress production of TH2 cytokines and are suppressors of allergic responses. The overall pharmacologic effects of these ligands include a decrease in the levels of IgE, inhibition of airway eosinophilia, and attenuation of airway hyperresponsiveness. The ligand appears to be beneficial for airway remodeling. This is due to inhibition of goblet cell hyperplasia, reduction in airway hyperreactivity, suppression of mucus production, and reduction of peribronchial smooth muscle layer thickening. There is also a decrease in the production of TGF-β1. CpG ODN reverses symptoms of atopic asthma when either used by itself or in combination with immunotherapy, in established asthmatic patients. The combination of a TLR2 agonist with allergens administered in the form of sublingual immunotherapy exhibits inhibition of airway hyperresponsiveness, which is accompanied by attenuation of TH2 responses.

7.9 Neural Pathways in Asthma

The pathophysiological changes in asthma may be modulated by sensory nervous receptors. These sensory nervous receptors are divided into three groups which include C-fiber receptors, the rapidly adapting receptors, and delta-nociceptive receptors. These receptors are activated by inflammatory and immunologic changes.

The axon reflex neurogenic inflammation–bronchoconstriction and mucus secretion may be mediated by the activation of C-fiber receptors. The activation of receptors causes reflexes, which include bronchoconstriction, mucus secretion, and mucosal vasodilation. The three basic reflex pathways are responsible for the cough reflex.

Adrenergic, cholinergic, and nonadrenergic–noncholinergic (NANC) nerves regulate the function of airways. Nervous system-mediated bronchoconstriction of the airways is produced by the cholinergic system. This is attributed to the neurotransmitter, acetylcholine, which mediates its effects via nicotinic and muscarinic receptors, and these actions are short lived. Acetylcholine also modulates its own release from postganglionic cholinergic nerves. The postganglionic nerves are home to M2 autoreceptors at prejunctions, which control the release of acetylcholine after their stimulation. Allergens, viruses, and ozone inhibit the activation of neuronal muscarinic M2 autoreceptors. The neuronal bronchodilatory mechanisms constitute inhibitory NANC (i-NANC) mechanisms, which utilize nitric oxide and vasoactive intestinal peptide (VIP) as their neurotransmitters. The excitatory part of the NANC employs substance P and neurokinin A as neurotransmitters, and smooth muscles of both large and small airways express neurokinin A-2 receptors. Tachykinins produce bronchoconstrictor effects by interacting with NK2 receptors, whereas NK1 receptors are involved in exhibiting the pro-inflammatory effects of substance P.

7.9.1 Role of Neuropeptides in Airway Inflammation

The stimulation of C-fibers by capsaicin causes a subset of sensory airway neurons to release several neuropeptides, which include tachykinin, substance P, and neurokinin A. In addition to capsaicin, other endogenous mediators including histamine, prostaglandins, and bradykinins can also result in their release. These neuropeptides are responsible for neurogenic inflammation, which is characterized by vasodilation, mucus secretion, plasma protein extravasation, increased expression of the adhesion molecules, and bronchoconstriction.

Neuropeptides are degraded by enzymes called neutral endopeptidases, which are present in adjacent to neuropeptide receptors. Neutral endopeptidases regulate the neuropeptide-induced responses by modulating their levels. Inhibitors of these enzymes are being studied as a potential therapeutic agent for asthmatic disease.

Besides neuropeptides, nitric oxide is another inflammatory mediator in the airways, which is also a vasodilator and a neurotransmitter. Nitric oxide is produced by the enzymatic action of nitric oxide synthetase on L-arginine. Airways contain this enzyme in three different forms; two of them which are termed neuronal and endothelial nitric oxide synthetase are constitutive, whereas the third form called inducible nitric oxide synthetase is inducible. The inflammatory cytokines including IL-1 and TNF-α augment the expression of inducible nitric oxide synthetase in human airway epithelial cells. Nitric oxide causes bronchodilation as a result of the relaxation of bronchial smooth muscles. It has also been suggested that nitric oxide is the neurotransmitter of the inhibitory nonadrenergic–noncholinergic bronchodilation.

The detrimental effects of nitric oxide include airway inflammation and vasodilation. It causes airway edema by increasing the erudition of plasma due to increased blood flow to postcapillary venules. The increased blood flow may also contribute to an increased mucus secretion.

Nitric oxide may suppress TH1 subset and its high levels may increase the expression of TH2 subset. The action of inducible nitric oxide synthetase may result in increased exhaled nitric oxide in asthmatics. Airway epithelial cells are the predominant source of increased nitric oxide in asthmatic subjects.

7.9.2 Neurotrophins in Asthma

The role of neurotrophins in the pathogenesis of allergic disease and asthma has only been recently appreciated. Neurotrophins are a family of growth factors, which have common receptors and physiologic effects and are the principal regulators of the neuronal activity, differentiation, and maintenance. In humans, there are at least four defined neurotrophins also termed as nerve growth factors. These polypeptides include nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF). They all act on a common group of tropomyosin-related tyrosine kinase (Trk) receptors. Neurotrophin receptors are expressed in the neurons of both the central and peripheral nervous systems.

Airway epithelia and alveolar macrophages constitutively express neurotrophins, and under normal conditions, low levels of neurotrophins are produced. However, in allergic inflammation, these levels rise considerably in atopic patients. After inhalation of an allergen by asthmatic patients, there are high concentrations of NGF, NT-3, and BDNF. The cellular sources of the increased neurotrophins include invading leukocytes and resident lung cells. Inflammatory cytokines IL-1 and TNF-α stimulate epithelial neurotrophin expression. The increased production of neurotrophins during allergic inflammation suggests a role of neurotrophins in asthma.

The allergic airway inflammation affects the neural reflex pathway and the function of afferent sensory nerves and synaptic transmission. Coughing and sneezing could result from protective axonal responses after activation of sensory airway nerves due to allergens. Inflammatory mediators also regulate the changes in airway sensory innervations. The neurotrophins affect allergic hyperresponsiveness by enhancing peptidergic sensory airway nerve function. Allergic asthmatic patients have higher blood levels of NGF. The influx of inflammatory cells in the lungs is observed as the neurotrophin expression is augmented. Multiple targets may be affected by neurotrophins as they play a role in allergic inflammation, which includes recruitment, maintenance, and activation of mast cells and eosinophils, as well as facilitation of TH2 response.

The neurotrophins play a role as the mediators of neurogenic inflammation. This is mediated by their effects both on the immune and nervous systems. The function of sensory neurons is regulated by neurotrophins. Furthermore, nerve growth factor

regulates expression of neuropeptide genes in adult sensory neurons. The release of neuropeptide substance P from neurons is augmented in allergic inflammation with contribution from neurotrophins, which are overproduced in this process. In parallel, the neurotrophin receptors are expressed on leukocytes, and consequently neurotrophins modulate neurogenic inflammation by at least two mechanisms. One mechanism of regulation is via controlling the extent and intensity of local immune response directly by activation of the neurotrophin receptors. The other possible mechanism of regulation of neurogenic inflammation by neurotrophins is via modulating the synthesis of neuropeptides in local neurons. The effects of neurotrophins are brief, self-limited, and in the vicinity of the area of their local synthesis. They possess a very short half-life but could be produced continuously, when the symptoms of allergic inflammation are manifested.

7.10 Airway Remodeling

The structural changes in the lungs of asthmatics resulting from airway inflammation and lung injury, followed by abnormal repair, are defined as airway remodeling. This is responsible for sub-phenotypes of asthma, which constitutes partially reversible or irreversible airway obstruction. There is also rapid decline in the function of the lungs. In fatal asthma the entire airway wall undergoes structural changes. The changes in airway remodeling are more pronounced in large membranous and small cartilaginous airways, although there are modifications in the entire bronchial tree. The patients with nonfatal asthma exhibit changes, which are localized and less prominent. In fatal asthma the thickening of the airway wall is more pronounced than nonfatal asthma. The airway wall thickening results from changes in epithelial cells, submucosal gland hyperplasia, subepithelial fibrosis, increased airway vascularization, and increased airway smooth muscle mass (Fig. 7.6).

Chronic inflammation that causes tissue injury produces structural changes as a result of the tissue repair. The asthmatic airway inflammation is deemed as the first step for airway remodeling. Increased production of TH2 cytokines is involved in the pathogenesis of airway remodeling. The overexpression of IL-4 and IL-5 causes subepithelial fibrosis, eosinophilia, and mucus metaplasia. However, there is some controversy regarding the exact role of IL-4 in these events. Enhanced secretion of IL-13 results in inflammatory infiltrates of eosinophils and macrophages, in addition to subepithelial fibrosis and mucus metaplasia. Mast cells and eosinophils play roles in the remodeling of epithelial cells. This is due to their effects on epithelial proliferation, barrier function, goblet cell formation, and epithelial desquamation. However, additional factors may contribute to airway remodeling since TH2 cell-independent nonatopic asthmatic patients also undergo airway remodeling. As a consequence, epithelial-driven models of airway remodeling have also been proposed that may explain the process in nonatopic or in all asthmatics. This concept is referred to as the model of the epithelial mesenchymal trophic unit or EMTU.

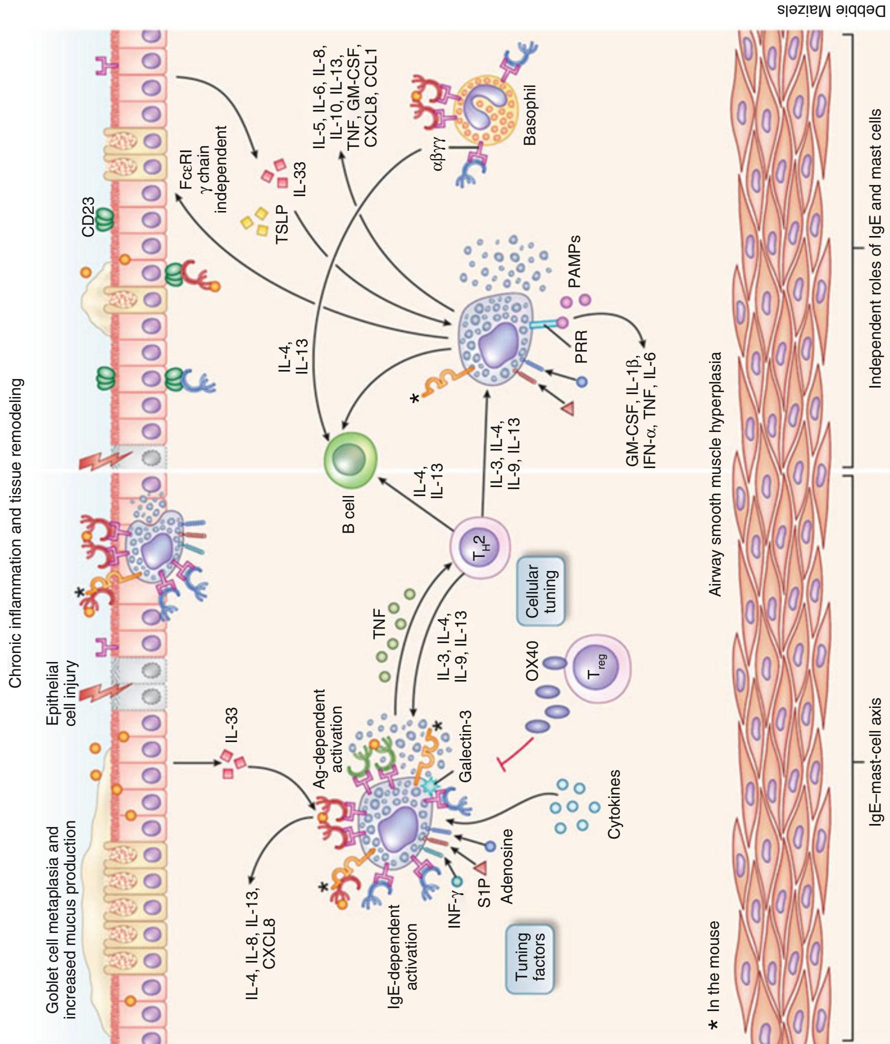

Fig. 7.6 Airway remodeling in asthma: The triggering of airway epithelial cells by proteolytic allergens, repeated viral infections, environmental pollutants, or TH2 cytokines results in the production of TGF-β and TGF-α. Neoangiogenesis and lymphoangiogenesis are the result of action of vascular endothelial growth factors A and C which are secreted by airway epithelial cells. Dendritic cells use new lymphatics to migrate to lymph nodes. There are also increased extravasations of inflammatory cells because of newly formed blood vessels. The proteolytic enzymes and cytokines interrupt epithelial tight junctions that allow the access of epidermal growth factor to its receptors. This causes induction of airway epithelial cells, and as a result there is release of periostin that enhances the secretion of TGF-β. The ultimate effect is differentiation of fibroblasts into myofibroblasts which synthesize collagen and extracellular matrix components (Reproduced with permission. Source: Lamrecht BN, Hammad H (2012) The airway epithelium in asthma. *Nature Med* 18:684–692. Nature Publishing Group)

According to this explanation, the etiology of asthma is based on a defective airway epithelium, both structurally and functionally. There is consistent environmental damage to a defective airway epithelium by infection, pollution, and cigarette smoke that causes airway inflammation, hyperresponsiveness, and remodeling. In animal models, innate responses to respiratory viral infections aid in the emergence of airway inflammation which is observed in asthma. The infection produces chronic inflammatory response accompanied by airway hyperresponsiveness and remodeling. Nonetheless, there is a correlation between the chronic response in animal models and inflammatory response in the airways of asthmatics. The extent of the chronic inflammatory state depends on the severity of the infection and is a product of an innate epithelial response. The response is characterized via activation of macrophages by natural killer T cells, which produce pro-inflammatory cytokines including IL-13. This causes chronic mucus cell metaplasia.

In asthmatic airways, there is shedding of epithelial cells, hyperplasia of goblet cells, and loss of ciliated cells. The patients with moderate to severe asthma exhibit thickening of the epithelium and subepithelial fibrosis. This is not the case with mild persistent asthmatics, chronic bronchitis patients, and healthy individuals. The data in this context is controversial, but suggests a role for proliferation of the epithelial cells in the thickening of airways.

There is support for EMTU hypothesis for the pathogenesis of asthma based on modifications exhibited in airway epithelium. The epithelium of asthmatic patients overexpresses epithelial growth factor receptors and MUC5AC (mucin glycoprotein), which are responsible for tissue repair after injury.

Subepithelial fibrosis contributes to airway remodeling. In asthmatics there is a thickening of an area just below the true basement membrane known as lamina reticularis. It appears to be the beginning phase of airway remodeling based on this observation in children and in adults. The concept is controversial since there are non- asthmatics with subepithelial fibrosis and some asthmatics lacking subepithelial fibrosis. Therefore, its functional aspects are not clear in airway remodeling. Nonetheless, thickening of the lamina reticularis supports EMTU model for the pathogenesis of airway remodeling in asthmatics. The latent and active forms of TGF-β exhibit augmented levels in response to changes in the epithelium. This response is even higher in patients with asthma as compared to normal controls. In lamina reticularis there is accumulation of fibrogenic factors, suggesting that this may serve as a signal transducer emanating from an innately defective epithelium and communicating to the inner tissues of the airway wall.

The most pronounced trait of airway remodeling is increased airway smooth muscle (ASM) mass. The increase in ASM mass is not comparable to the increase in the thickness of the wall. The increase in ASM mass results from an increase in cell size, cell numbers, and probable migration of other cell types to the ASM bundles. The data for increased number is well supported, whereas the contribution of cell mass in increase in ASM mass is questionable. A number of endogenous mediators including autacoids and cytokines could play a role in increasing the muscle mass by being mitogenic. These endogenous mediators include IL-1β, IL-6, TGF-β, histamine, serotonin, leukotrienes, vascular endothelial growth factor (VEGF),

tryptase, and thromboxanes, and others have also been found in airway biopsies. Their receptor systems regulate the phosphoinositide 3′-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways. The transcription factors activated by ERK and PI3K phosphorylate cyclins (type D) induce the cell cycle, resulting in the proliferation of ASM. The structural changes resulting from airway remodeling produce sub-phenotypes of the symptoms of asthma and are associated with the increased severity of the disease.

7.11 Role of Mitogen-Activated Protein Kinases (MAPKs) in Asthma

MAPKs are involved in multiple signaling pathways during inflammation, immune response, apoptosis, and cell proliferation. As a consequence, they are involved in many aspects of the pathogenesis of asthma. The MAPKs are classified into six classes, ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3, and p38 (α/β/γ/δ), and play a role in various cell functions. ERK1/2 signaling is involved in the pathogenesis of asthma. The allergic inflammation in the airways is attenuated by MAPK antagonists and is inhibited in MAPK knockout mice. The patients with atopic asthma have increased expression of phosphorylated ERK1/2, p-38, and JNK 1/2/3 that correlates with their clinical manifestation. Furthermore, ERK1-/ERK2-induced expression of JunB and sprouty-2 is augmented in the airways of asthmatic patients. JunB plays a role in the differentiation of TH2 cells. Sprouty-2 is involved in the development of the lung and induces ERK1/2. There is an increase particularly in the airway epithelium of asthmatic patients of both ERK1/2 and Sprouty-2. A correlation exists between the expression of ERK1/2 and the numbers of circulating eosinophils and neutrophils. The levels of ERK2 are higher in lymphoid and myeloid tissue, whereas the opposite is the case for the lung tissue.

The MAPK signaling plays a role in development of $CD4^+$ and $CD8^+$ T cells from naïve helper T cells. The retention in the thymus of helper and cytolytic T cells is mediated via ERK1/2, whereas their clonal deletion is a result of action of JNK and p38. This suggests that TH2 differentiation is promoted by ERK1/2, and it is required for the development of helper T cells. There is evidence that $CD4^+$ T cells lacking ERK2 produce suppression of the differentiation of CD4 precursors. The antagonists of ERK1/2 support the development of cytolytic T cells. The inflammatory responses of asthma are suppressed in animal models having ERK1-deficient T cells. In both acute and chronic mouse models of asthma, ERK1 deficiency produces inhibition of airway inflammation and hyperactivity. This is accompanied by lower levels of IL-4 and IL-5, without any effect on IL-17A or IFN-γ. ERK1-deficient T cells also have lower expression of JunB. TH2 differentiation requires IL-4 binding to its promoter, which is mediated by JunB in response to ERK1 (Fig. 7.7).

Alveolar macrophages contribute to the pathogenesis of asthma. p38 is important and is involved in the secretion of IL-1β, TNF-α, cyclooxygenase 2, and inducible nitric oxide synthase. There is evidence that ERK1/2 and p38 are involved in both

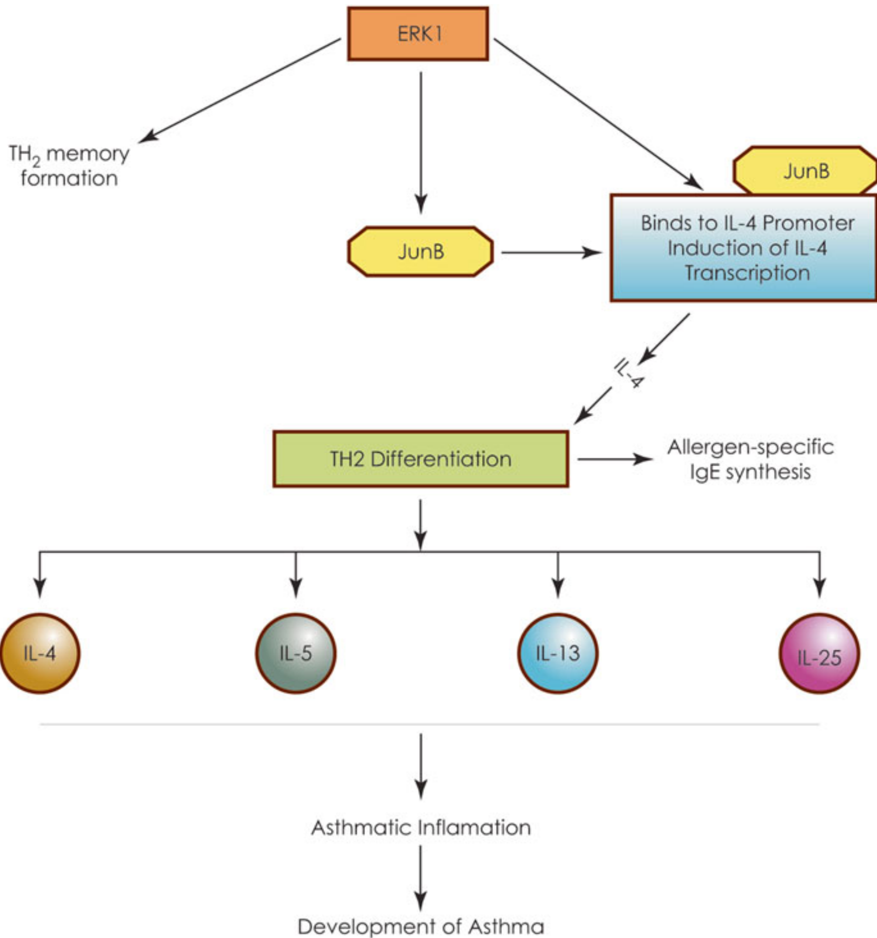

Fig. 7.7 Role of ERK1 in asthma. ERK1 signaling is required for the development of TH2 responses, resulting in asthmatic inflammation. ERK1 expression causes the maintenance of the expression of JunB, which binds to IL-4 promoter. This causes the transcription of IL-4. The ultimate effect is TH2 cell differentiation, synthesis of TH2 cytokines, and allergen-specific IgE synthesis (Adopted from Goplen N. et al. *FASEB Journal* 2012, 26: 1934–1945 (Reproduced with permission. Source: Khan MM, Peterson EB (2015) The role of Mitogen-Activated Protein Kinases in Asthma. *Curr Immunol Rev* 11:132–146. Bentham Publishers)

the pro-inflammatory and anti-inflammatory responses in asthmatics. Both ERK1/2 and p38 are also involved in the function of mast cells. ERK1/2 exhibits a role in the survival of mast cells, while p38-dependent mechanisms dictate the migration of mast cells. JNK along with ERK1/2 produces GM-CSF and eicosanoids from mast cells. MEK2-deficient mast cells lack the ability to secrete IL-4, IL-6, and TNF-α. In allergic asthma migration of eosinophils into the lungs is crucial, and ERK1/2 and p38 activate and differentiate eosinophils in addition to other biological pathways.

ERK1/2 plays a role in the priming, movement, endogenous mediator production, cytokine secretion, and degranulation of human eosinophils. There is an increase in the activation of ERK1/2 in eosinophils isolated from the airways of asthmatic patients. P38 also plays a role in the differentiation of eosinophils from stem cells, as well as in their migration, degranulation, and cytokine production. The growth factor-dependent Ras-mediated activation of eosinophils is inhibited by Spred-1, which regulates the proliferation and function of eosinophils in response to IL-5.

Dendritic cells, another player in allergic disease/asthma, are also regulated by MAPK. p38 in dendritic cells plays a role in migration of these cells, increased expression of HLA Class II molecules, and upregulation of co-stimulatory molecules, involved in antigen presentation. In animal models, dendritic cell function correlates with the strength and duration of p38 signaling. The migration of dendritic cells is the product of a weak and transient p38 signal, whereas cytokines are produced by a strong and continuous p38 signal. ERK1/2 regulates dendritic cells by induction of IL-10 secretion and suppression of IL-12 production. Generally, the cytokine secretion from dendritic cells reflects the ratio of the degree of ERK1/2 and p38 activation. Enhanced ERK1/2 levels produce TH2 response, as there is increased IL-6 and IL-10 secretion and inhibition of IL-12 production.

MAPK also play a role in the regulation of airway epithelial cells, which provide an important link between innate immunity and allergy through TLR. In epithelial cells both ERK1/2 and p38 modulate RANTES and eotaxin-3 secretion. However, IL-8 production is only regulated by p38 and not by ERK1/2. MAPK13, an isoform of p38 ŏ, produces IL-13-induced mucus production and leads to activation of ELK1 and ATF2. In human bronchial epithelial walls, JNK activation causes LPA-induced IL-13 decoy receptor activation. The airway remodeling caused by house dust mite is dependent on the induction of isozyme JNK1.

The role of airway smooth muscle (ASM) cells is important in the obstruction of airways. Airway smooth muscle cells express high-affinity (FcεRI) and low-affinity (FcεRII) IgE-Fc receptors, and IgE produces abnormal smooth muscle contraction. There is also sequential autocrine release of IL-4, IL-5, and IL-13, but not IFN-ϒ, in response to sensitization of airway smooth muscle cells by IgE. In airway smooth muscle cells isolated from the bronchial tissue of asthmatic patients, the secretion of IL-6, IL-8, and TNF-α via ERK1/2 and p38 MAP kinases is augmented. Airway smooth muscle cells also express chemokines CCR1 and CCR3. It has been proposed that IgE mediates CCL15 production via stimulation of airway smooth muscle cells. In response to IgE, airway smooth muscle cells will release IL-6, IL-8, and TNF-α. The presence of TNF-α will cause the production of CCL15 from airway smooth muscle cells. There is a synergistic enhancement of this effect in the presence of IFN-ϒ. The severity and persistent of asthma may also be a consequence of the binding of CCL15 to CCR1. The expression of CCR1 on airway smooth muscle cells is induced by IFN-ϒ and TNF-α. ERK1/2 indirectly affects the function of airway smooth muscle cells via regulating the secretion of various cytokines and chemokines. ASM hyperplasia requires the Ras/Raf/ERK transduction pathway. Furthermore, continuous activation of ERK1/2 results in the functioning of the EGF (epidermal growth factor), PDGF (platelet-derived growth factor), and thrombin in

human ASM. In airway muscle cells, the expression of cyclin D is dependent on ERK1/2 that is required for the progression of the cell cycle. The ultimate effect is the increased ASM mass in patients with asthma. Furthermore, ERK1/2, p38, and JNK signaling pathways are involved in IL-17A-induced eotaxin-1/CCL11 expression in ASM cells. CCL11 selectively recruits eosinophils.

The role of ERK5 in lung disease has also been investigated and found to be associated with lung fibrosis. ERK5-mediated effects involve TGF-β1-Smad3 cascade and regulation of Smad3 acetylation. This results in increased extracellular matrix production, which is also a feature of airway remodeling in chronic asthma.

7.12 Mitogen-Activated Protein Kinase Bistability

The system stimulation of a cellular process is sustained or prolonged after the initial input. There is a need of at least two possible stable states for sustained signaling. The stimulus–response relationships could be linear, rectangular, hyperbola, or sigmoidal. The phenomenon of ultrasensitivity is observed in sigmoidal stimulus–response; in this case a small change in input produces a major change in the output response. The ultrasensitivity characteristics are exhibited by MAPK signaling, in reference to MEK protein kinase catalyzing phosphorylation as well as at two phosphorylation sites of MAPK p42 ERK. In this case MEK is dissociated from dual phosphorylated substrate after phosphorylating both sites 1 and 2. The phosphorylation of the second site threonine is essential for the activation of pMAPK to phosphorylate downstream substrates, after MAPK becomes phosphorylated through MEK. This is not accomplished by a single ultrasensitive mechanism; rather ultrasensitivity could only be achieved after an activation threshold is reached.

The cellular phenotype of bistability, nevertheless, is observed in cellular signaling with ultrasensitive character. It entails the ability of a cell to function in a switch-like manner that can be turned on or off and is in response to a graded stimulus. This suggests the existence of all or none response during different cellular states. The cell determines its own dominant state from the two based on its environment. There are control mechanisms including feedback control, which render bistability in a cell. In MAPK pathways positive feedback loops are present, which is the case where JNK activates MAPKKK.

The cellular memory may also be the product of bistability, involving autophosphorylating protein kinase. In lymphocytes there is evidence of hysteresis based on positive feedback loop. In this case there is conversion of analog input to digital output in studies using SOS-mediated Ras activation. Bistability plays a role in the persistence of asthma as evident by the effects of IL-13 on ERK1/2 phosphorylation in epithelial cells. In this case intracellular adaptor Sprouty 2 serves as a positive modulator of ERK1/2 action. In addition, mitogen-activated protein kinase-interacting kinase 1 (Mnk1) perhaps phosphorylates Sprouty 2 to prolong the signaling of ERK1/2. This results in prolonged cytokine and chemokine secretion (Fig. 7.8).

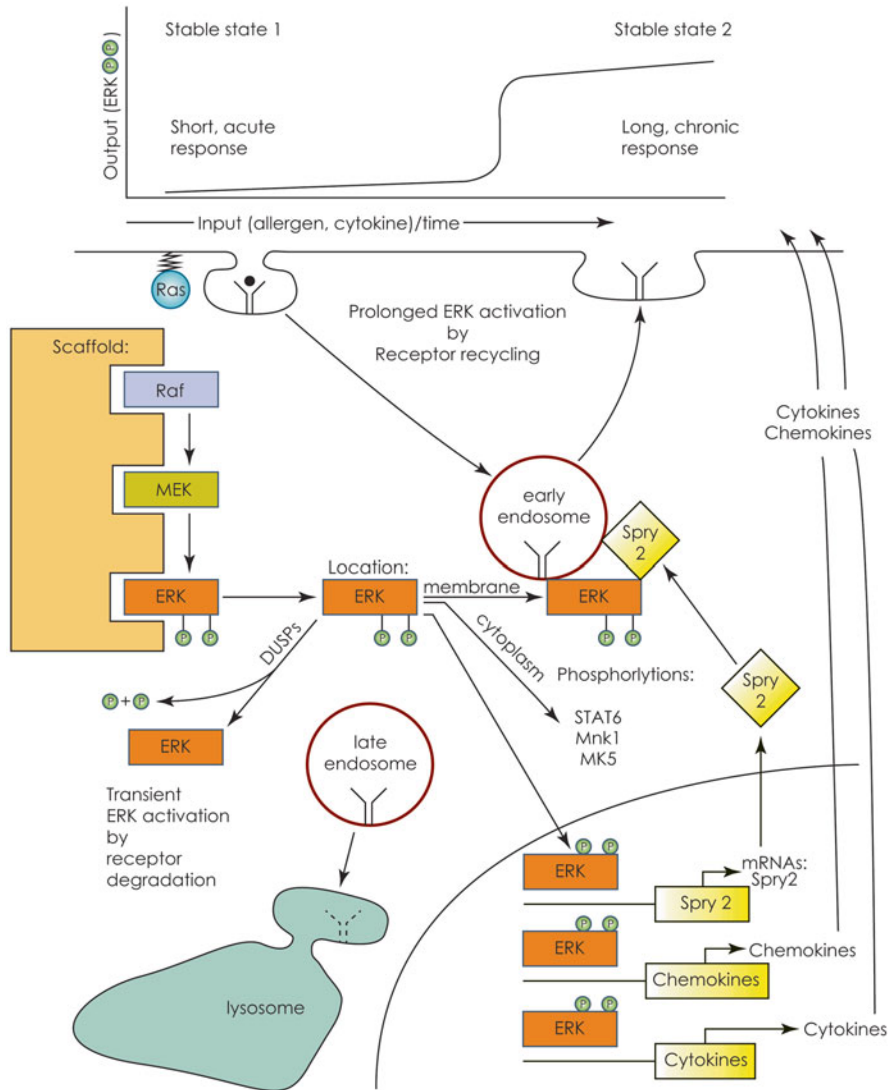

Fig. 7.8 MAPK bistability: Phosphorylation of bottom-tier MAPK, such as ERK1/2, reflects the function of extracellular stimuli, including allergens and pro-inflammatory cytokine, and additional exposures over time and are used to measure the cellular response. The effect of pro-inflammatory stimuli on initial cellular response results in ERK1-/ERK2-associated cellular response of a transient nature, which is associated with stable state 1. This is the result of receptor endocytosis and preferential receptor degradation. Repeated exposure to pro-inflammatory stimuli causes induction of adaptor Sprouty2, which prolongs ERK activation by recycling responding receptor and not causing its degradation, and as a result promoting stable state 2 acts at early endosomes in the presence of active ERK1/2. The subsequent cellular response causes enhanced production and secretion of cytokines and chemokines to attract more pro-inflammatory lymphocytes and leukocytes to the inflammatory site (Adapted from Liu et al. (2008). Reproduced with permission. Contributed by Eric B. Patterson in: Khan MM, Patterson EB (2015) The role of Mitogen-Activated Protein Kinases in Asthma. *Curr Immunol Rev* 11:132–146. Bentham Publishers)

A memory-driven process is responsible for the continuous symptoms of asthma and hyperresponsiveness in some patients. External factors include not only environmental allergens but cytokines released continuously as a result of repeated and cumulative input of allergens or cytokines over time. Repeated cytokine stimulation leads to augmented expression of multiple cytokines, via ERK1/2 signal bistability, functioning in the "On" stable switch mode. Cytokine receptor recycling causes release of agents to sustain asthma pathology, rather than cytokine receptor degradation to decrease pathologic tissue response. As a result, the cellular events provide an explanation for the persistent airway hyperresponsiveness and asthma symptoms initiated by external factors but sustained by the constitutive expression of cellular cytokines.

This memory constitutes a combination of immunologic and tissue-based memory. Immunologic memory resides in memory T and B cells, whereas tissue-based memory perhaps is the product of sustained signaling. The sustained kinase signaling is responsible for pERK1-/pERK2-induced chronic asthma, where this sustenance results from repeated exposure to TH2 cytokines. This is an example of ERK1/2 bistability and may explain resistance to glucocorticoids in chronic asthmatics. Furthermore, this would explain that in chronic asthmatics undergoing allergen-specific immunotherapy, there is no reduction in their symptoms despite very significant immunologic changes and reduced skin sensitivity. The asthma is maintained despite reduction in sensitivity to allergens. There is MAPK activation in chronic asthmatics. Human T cells resistant to TGF-β, IL-10, and glucocorticoid suppression proliferate in response to IL-2 and IL-4, accompanied by increased expression of MEK1 and enhanced ERK1/2 function. Antagonism of MEK1 restores TGF-β and IL-10 activity, highlighting a therapeutic option for MEK1 antagonists.

The role of T-cell receptor in asthma is also of interest. A digital response is observed in response to TCR superantigen staphylococcal enterotoxin (SEE) input, when pERK output is measured. In contrast another model produced an analog response. Scaffold protein KSR1 is an important contributor of the MAPK signaling output that regulates the sensitivity but not the quality of the T-cell response. Mitogen-activated T cells produce an overall digital response. Different activation qualities are exhibited by various factors required for IL-2 transcription, such as NFATc2 and NF-kB. The translocation of NFATc2 utilizes digital mode, whereas NF-kB translocation employs analog mode. But there are differences depending on the stimulus, such as digital NF-kB activation is observed when anti-CD3 is used. This suggests that signaling system outputs throughout the transduction pathway need to be considered for clinical interventions to treat asthma. This will include targeting cell surface receptor activation, intermediate MAPK module activation, migration to the nucleus, and the resulting transcription.

For the MAPK activity from cell surface receptors to downstream signaling, various cytoplasmic proteins called adaptors, docking proteins, and scaffolds are required. These proteins may undergo phosphorylation or dephosphorylation for their activation or deactivation and are involved in the downstream signaling activities of MAPK. For the clinical utility of the concept of bistability, it is important to understand the role of plasma membrane-associated proteins and cytoplasmic scaffold proteins in reference to cellular bistability and digital signaling responses.

The role of Ras, MAPKKK C-Raf, and KSR has been identified in such signaling. Adopter proteins, which are of low molecular weight, are involved in sustained MAPK signaling, MAPK output, and sustained signaling. One adopter protein, Sprouty 2, is involved in IL-13-induced ERK1/2 phosphorylation and signaling in the lung epithelial cells. This bistable signaling is associated with Rab5+ endosomes. An increased ERK1/2 phosphorylation and sustained MAPK signaling can be achieved by a positive feedback loop, resulting from enhanced and persistent IL-13 receptor recycling. The binding of the cytokine to the receptor on a cell induces MAPK module activation and ERK1/2 phosphorylation. The downstream cytoplasmic targets are phosphorylated by ERK, resulting in the transcription of cytokines, chemokines, and Sprouty 2 gene. This would result in sustained MAPK activation state and asthma symptoms through cytokine receptor recycling, where the gene transcription is associated with active ERK1/2 and Rab5+ endosomes. Additional roles are played by docking and scaffold proteins. Gab docking proteins play a role in the response of leukocytes to cytokines. MAPK uses scaffolding platforms to aggregate kinase activities. The activation of transduction pathways after binding of a ligand to its receptors results in intracellular scaffold recruitment, which enhances the efficiency of signal transmission. β-Arrestins play a role as a scaffolding activity for the ERK1/2, MEK1, and c-raf module and thus influence MAPK signaling. Deletion of β-arrestin 2 gene attenuates the migration of T cells to lung airways and inhibits symptoms of lung inflammation.

7.13 Future Treatment Options

The conventional drugs have proved to be ineffective in treating many cases of asthma. A number of patients have uncontrolled or partially controlled asthma, which negatively impacts their quality of life. The diversity of human asthma presents a challenge to successfully treat it. At present, asthmatic inflammation could most effectively be treated with glucocorticoids. They suppress the expression of inflammatory genes (pro-inflammatory cytokines), produce anti-inflammatory activity in the lung, and cause a decrease in the number of exacerbations. But asthma is not a one disease and in some patients glucocorticoids have limited value. Treating corticosteroid refractory asthma is a challenge and is associated with high morbidity and mortality. One treatment is the use of omalizumab, which is a specific antibody for IgE. Another monoclonal antibody, keliximab, which targets CD4 receptors has not yet been approved for clinical use in the United States, and based on the data, it is not very promising. It reduces the number of T cells and allergen-induced T-cell proliferation but has limited effects on asthma endpoints. Another approach has been the targeting of IL-4Rα. There is a consensus that IL-4-directed therapies by themselves may not be successful due to the fact that IL-4 and IL-13 are redundant in their physiologic effects. This leads to the idea of targeting both IL-4 and IL-13 at the same time. Since IL-4Rα is shared between IL-4 and IL-13 for the activation of STAT6 signaling pathway, blocking this receptor may have beneficial effects to treat asthma. Pitrakinra, a recombinant modified IL-4Rα agonist, with two

functional mutations blocks both IL-4- and IL-13-dependent signal transduction pathways. This IL-4 receptor agonist is undergoing clinical trials for the late-phase response after allergen challenge in atopic asthmatics. Pitrakinra suppressed the inhibition in FEV_1 against the allergen challenge, but better efficacy was observed by inhalation dosing. These observations pointed out that the lung is the primary site of action of this potential drug. However, the drug is selective in only a subset of patients of asthma. Dupilumab, a monoclonal antibody directed against IL-4Rα, modulates signaling of both the IL-4 and IL-13 pathways, is under clinical development, and has shown 87 % reduction in the exacerbation of asthma in moderate to severe atopic asthmatic patients. Mepolizumab is a humanized monoclonal antibody, which is directed against IL-5 and has very recently been approved for the treatment of severe eosinophilic asthma.

Enokizumab is a humanized monoclonal antibody which is directed against IL-9 and is designed to treat asthma. The drug has not yet been approved for treatment in the United States. Lebrikizumab, a humanized anti-IL-13 monoclonal antibody, significantly improves lung function in a subset of patients of asthma, where their symptoms are not satisfactorily managed with inhaled corticosteroids. It significantly decreases amount of fractional exhaled nitric oxide, indicating inhibition of airway inflammation. The effects of lebrikizumab in patients receiving inhaled corticosteroids and not receiving inhaled corticosteroids were variable, specifically for the lung function. The findings show that the treatment with lebrikizumab is most efficacious in asthmatics receiving inhaled corticosteroids who have high serum levels of periostin. Periostin is a signaling protein expressed by the epithelial cells of the bronchi and IL-13 is required for its induction. It has a role in airway hyperresponsiveness, inflammation, and in airway remodeling. Inhibiting IL-13 in patients not receiving inhaled corticosteroids is not enough to increase FEV1 when compared with the standard treatment. As a consequence, the selection of the subset of asthmatic patients will be important for treatment with lebrikizumab. When discussing antibody to IL-13, it is important to point out that IL-13 responsive genes are expressed in the airway epithelial cells of only 50 % of asthmatic patients and are associated with a strong TH2 response in those biopsies. This is termed as "TH2 high" as opposed to "TH2 low." In "TH2-low" asthmatics, IL-13-responsive gene expression signature is close to the normal control subjects. There is increased airway hyperresponsiveness, eosinophilia, serum IgE levels, and IL-5 and IL-13 expression (bronchial biopsies) in TH2-high asthmatics. Other monoclonal antibodies directed against IL-13, and under investigation, include anrukinzumab and tralokinumab.

Daclizumab has also been investigated for asthma but the benefits to side effects ratio are negative. Other options with limited clinical utility and with serious side effects include cyclosporine, tacrolimus, azathioprine, methotrexate, and intravenous immunoglobulins. Antibody against CC chemokine receptor 4 (CCR4) is under early phases of clinical trial for asthma although it has been approved for relapsed or refractory adult T-cell leukemia/lymphoma in Japan. Its development for the treatment of asthma is based on the observation that release of CCL17 and CCL22 recruits TH2 cells into the airways after their release from epithelial cells. They bind to TH2 cells via their receptors, CCR4.

Induction of Treg cells may provide a novel approach for the treatment of asthma. This could potentially be accomplished by delivering modified *Foxp3* mRNA to sites of inflammation and may treat asthma and other TH2- and IL-17-/IL-23-mediated conditions. A site-specific, high-pressure intratracheal spray delivery of modified *Foxp3* mRNA to the lung that protects against airway hyperresponsiveness and airway inflammation in vivo is of interest. This is a result of modulating both TH2 and TH17 responses in an IL-10-dependent fashion.

Another way of inhibiting the IL-4Rα expression and as a consequence IL-4 and IL-13 signaling is the use of antisense oligonucleotides. One investigational agent did not show much promise. In preclinical settings, compounds have been tested to block STAT6 activity or STAT6 expression. In these studies STAT6 inhibitory peptides, small interfering RNA (siRNA), and antisense RNA were employed.

Other agents currently under development include agents targeting MAPK and MAPK-associated proteins. A selective potent inhibitor of MEK1/2 produces suppression of allergen-specific increases in leukocyte count in the airways and the levels of IL-4, IL-5, and IL-13 in animal models. This compound also inhibited allergen-specific IgE production, adhesion molecule (VCAM-1) expression in the lung, and the mucus production in the airways. Other inhibitors of MAPK inhibited OVA-induced anaphylactic bronchial contraction and secretion of leukotrienes from lung fragments and influx of eosinophils. Using a combination of glucocorticoid with a MAPK inhibitor significantly alleviated lung inflammation and airway remodeling. Therefore, MAPK inhibitors may be important pharmacologic agents to treat asthmatic disease. Furthermore, the concept of bistability explains that some patients undergoing immunotherapy do not see improvement in their symptoms despite their IgE levels and cell count returning to normal. As a consequence, additional studies will be required before new treatments are developed based on the information gained by the bistability in asthma. As discussed, asthma is a complex disease, and multiple pathways are involved in its development and maintenance, which suggest that therapeutic interventions will require a multitude of strategies involving various receptors, activation/inhibition of genes, and exploring novel therapeutic targets.

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Chapter 8
Tissue Transplantation

Abstract In transplantation, organs, cells, or tissue is harvested from one individual and grafted into another individual. The transplant could be autologous, where the transplanted graft is from the same individual or it could be allogeneic, where the graft is transplanted in a genetically different individual. In this chapter, the focus is predominantly on allogeneic transplantation. The ability to distinguish self from nonself resides in the HLA molecules, which are identified for the purpose of tissue typing to find a compatible donor when necessary. Recognition of grafted tissue as self or nonself is the result of inheritance of codominant genes. The mechanism of rejection involves both the innate and acquired immune responses, and various cell types and molecules particularly T cells, NK cells, adhesion molecules, and antibodies have roles in these processes. This forms a significant part of the discussion. Lastly, transplantation of individual tissue and cell types including the kidney, liver, pancreas, heart, lung, hematopoietic stem cells, and bone marrow is described. There is discussion of graft-versus-host disease and ways to minimize the rejection process.

Keywords Chronic graft-versus-host disease • Leukemias • Lymphomas • Neuroblastoma • Gliomas • Myelodysplastic syndromes • Thalassemia • Aplastic anemia • Immune deficiency syndromes • IL-1β IL-18 • RAGE • IL-12 • Dendritic cells • Danger signal • Hyperacute rejection • Acute rejection • Chronic rejection • Tolerance • Plasmapheresis • CTLA-4 • CD28$^+$ T cell • B7 • HLA-A • HLA-B • HLA-D • HLA-DR • HLA-DP • HLA-D • Crossmatch • Graft-versus-host disease • IL-10 • IL-6 • Kidney transplantation • Liver transplantation • Pancreas transplantation • Heart transplantation • Lung transplantation • Hematopoietic stem cell transplantation • Bone marrow transplantation • Graft-versus-host disease • Acute graft-versus-host disease • Autograft • Allograft • Tissue typing • HLA typing • Major histocompatibility antigens • Minor histocompatibility antigens • T cells • CD4$^+$ cells • CD8$^+$ cells • NK cells • Immune effector cells • Monocytes • Cytokines • IFN-γ • TNF-α • TNF-β • Afferent phase • Central phase • Efferent phase • IL-2 • Effector cells • Memory T cells • Pattern recognition receptors • TLR • NOD-like receptors • RIG-like helicases • NALP3-inflammasome • Neutropenic phase

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• Systemic sclerosis • Multiple sclerosis • Rheumatoid arthritis • Juvenile idiopathic arthritis • Crohn's disease • Type I diabetes • Autoimmune-related retinopathy and optic neuropathy • Autoimmune-related retinitis and optic neuritis • Chronic inflammatory demyelinating polyneuropathy • Celiac disease • Neurovascular Sjögren syndrome and dermatomyositis/polymyositis • Stem cell transplantation in nonmalignant disease

8.1 Introduction

The concept of transplantation may go back to the stories of chimeras including sphinxes and mermaids. According to a third-century legend of Saints Cosmas and Damian, these two brother physicians replaced the cancerous leg of a church sacristan using the limb of a dead Moor. It is suggested that about 2000 years ago, transplantation surgery was done for nose reconstruction by Vaidya, the Ayurvedic physicians. Gaspare Tagliacozzi in the late sixteenth century performed reconstructive rhinoplasty by using skin from the arms of the patients. The allografts of skin always failed and no further progress was reported until the twentieth century.

The original experiments of tissue transplantation were done by using skin transplants in inbred strains of mice. In 1908, Alexis Carrel first reported kidney transplantation in cats, but all the cats died, and only some exhibited a functional urinary output. George Schone published in 1912 the difficulties associated with tissue transplantation, which were not related to surgery, asepsis, and anesthesia. He wrote six laws of tissue transplantation, which to date are accepted:

1. Transplanting a tissue to a different species fails.
2. Transplants in unrelated species members fail.
3. Autografts succeed.
4. First allograft is initially accepted but is rejected later.
5. Second allograft is rejected rapidly.
6. Close relationship between the donor and recipient aids the acceptance of the graft.

In 1935, a Russian surgeon performed the first human kidney transplant but the patient was not able to have kidney function and died. According to reports in 1954, the first successful kidney transplant was performed in the United States, where the donated organ was from an identical twin. Today tissue transplantation is a form of medical therapy where a failing or defective organ is replaced. The development of modern surgical techniques has helped overcome many obstacles facing this unique form of therapy. The other obstacle, the availability of donor organs, remains more in some cases than others.

However, the most serious obstacle to tissue transplantation is the immune response, specifically the acquired immunity. The blood transfusion provided the early understanding of the difficulties associated with this therapy. Considering the fact that there are only a few blood types, the complexity of the tissue type results in a major immune response against the grafted tissue. Both the blood and tissue

types are important for matching an organ for the transplant. Matching of blood type is important because of the expression of blood group antigens on vascular endothelium.

Several basic rules of tissue transplantation were established using skin transplantation in inbred strains of mice. Autografts, the transplantation of skin from one part of the body to another or between genetically identical animals or individuals, survive. Allogeneic graft, the transplantation of skin between nonidentical animals and individuals, is rejected. This rejection is T-cell dependent and is not exhibited by nude mice. The rejection after the first grafting of the tissue is called first-set rejection, and if the same recipient receives a second graft from the same donor, the rejection process is more rapid and is called second-set rejection. This rapid rejection is not observed when the graft is from a different donor.

The above-mentioned observations were also described in humans by P.D. Medawar in the 1940s. Based on his observations in humans, and later in animal experiments, he concluded that the graft rejection was a result of immune response, which eventually led to the discovery of major histocompatibility complex. The development of acquired immune response to transplanted tissue results in the rejection of the tissue where cytotoxic T cells, inflammatory T cells, and antibodies play a major role in the rejection process.

8.2 Tissue Typing in Transplantation

After tissue transplantation, the severity and the period of rejection depend on the tissue type, and this process involves the specificity and memory components of the immune response. Avrion Mitchison in the 1950s observed that allograft immunity could be transferred by the components of the cellular immune response, and antibodies present in the serum that were part of the humoral response were not associated with this process. Future studies delineated the role of T lymphocytes in the allograft rejection process, and the role of both $CD4^+$ and $CD8^+$ cells was established.

The differences in HLA are responsible for the initiation of an immune response against the nonself HLA molecules. Tissues with similar HLA types are designated as histocompatible, whereas histoincompatibility induces immune responses resulting in allograft rejection. Consequently, the matching of the HLA types between donor and recipient is instrumental in controlling the rejection, but it does not prevent it, which could be attributed to the polymorphism and complexity of the HLA. The HLA-identical unrelated individuals mostly do not possess identical MHC genotypes. As expected, this will not be an issue with HLA-identical siblings, but still the grafts between the HLA-identical siblings are slowly rejected with the exception of identical twins.

The HLA tissue typing plays an important role before the suitability of a donor and recipient could be determined. The first test is the matching of the blood groups, followed by HLA typing, which is done by microcytotoxicity test, which is per-

formed by using antibodies to the HLA Class I and HLA Class II molecules in microtiter plates. Various classes of the HLA Class I and HLA Class II molecules are used, complement is added following an incubation period, and cytotoxicity is measured by using one of the various dyes. If the monoclonal antibody is recognized by the antigen (HLA molecule) on the cell surface, the cell will then by lysed by the complement resulting in the uptake of the dye by the dead cells.

In addition to the major histocompatibility antigens, minor histocompatibility antigens (minor H antigens) are also involved in tissue rejection. However, in this case, graft rejection is slow and is the result of tissue transplantation where the donor and recipients differ at genetic loci other than the HLA. This response is much weaker because of a low number of T lymphocytes involved in this process. It has been reported that most minor H antigens may be bound to self HLA Class I molecules since $CD8^+$ T cells respond to these antigens. Proteasomes in cytoplasm digest self-proteins, and subsequently, digested proteins are transported to rough endoplasmic reticulum where they bind to the HLA Class I molecules and are expressed on the cell surface. If these expressed peptides are nonself as is the case in tissue transplantation, they will be recognized by T cells as foreign and an immune response will pursue. The response to minor H antigen resembles to immune response produced against the viral infections. Expression of minor H antigens on grafted cells causes the destruction of the graft.

8.3 Mechanisms of Rejection in Tissue Transplantation

The rejection of a transplant principally emanates from a cell-mediated immune response, which is specific for alloantigens that are primarily HLA molecules expressed on the cells of the transplanted tissue. It involves delayed-type hypersensitivity and cell-mediated cytotoxic reactions and can be divided into various distinct phases which are shown in Fig. 8.1. During the first phase, often defined by some as the afferent phase, the antigenic material shed by the graft is transported through the lymphatics to the draining lymph nodes. The antigens from the graft undergo uptake, processing, and presentation in context of HLA molecules by the antigen-presenting cells. The alloantigens of the graft are presented to both CD4+ and CD8+ cells, which after recognition proliferate in response to the antigen. Although both minor and major HLA antigens are recognized, minor histocompatibility antigens cause a weak response. However, a strong response is observed when it is the result of a combination of several minor histocompatibility antigens. The response to the major histocompatibility antigens involves recognizing HLA molecule of the grafted tissue and associated peptide ligand in HLA molecules cleft. The allogeneic cells produce proteins that form the grooves of the HLA Class I molecules of the donor. In contrast, the allogeneic cells do not synthesize proteins that are present in the grooves of the HLA Class II molecule; instead, they are the result of uptake and processing.

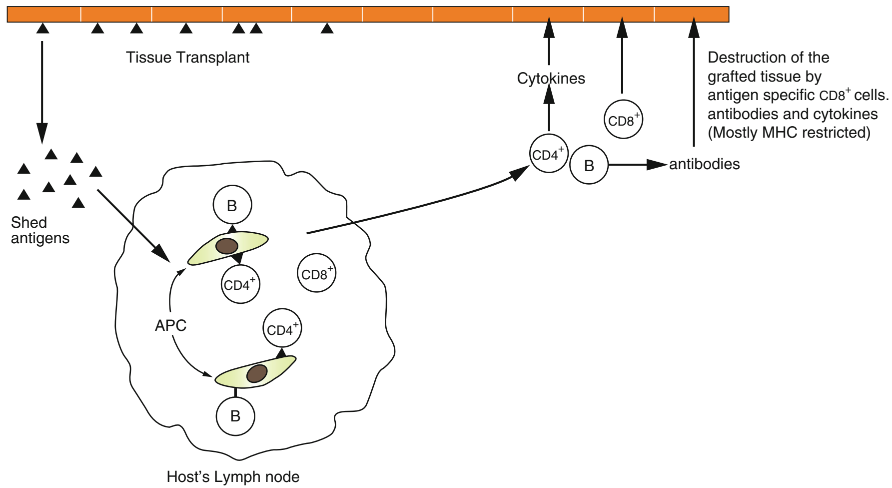

Fig. 8.1 Phases of tissue transplant rejection: the transplanted tissue shed antigens. These antigens undergo uptake, processing, and presentation to the T cells in the secondary lymphoid tissue by antigen-presenting cells which include macrophages, B cells, Langerhans cells, or dendritic cells. This phase results in the production of antibodies and antigen-specific TH and Tc cells. The antibodies and effector cells then migrate to the grafted tissue where TH cells secrete cytokines and which in combination with the antibodies and Tc cells destroy the grafted tissue

The next phase, also referred to as the central phase, involves the activation of the immune effector cells. When lymph nodes are examined following a tissue transplant, there is increased traffic of lymphocytes across the high endothelium of postcapillary venules. This is followed by the activation of TH cells, which results from the recognition of an alloantigen on the antigen-presenting cells in context of HLA molecules and required costimulatory signals. The antigen-presenting cells vary according to the transplanted organ but dendritic cells generally serve as antigen-presenting cells for most grafts. This is due to their ubiquitous distribution with high expression of the HLA Class II molecules. During this phase, there is also the development of lymphoid follicles and generation of graft-specific cytolytic T cells. The antigen-presenting cells of the host may also enter the graft and then endocytose both of its major and minor histocompatibility alloantigens and present them to the T cells in context of HLA molecules and the required costimulatory signals. There is a rapid proliferation of T cells in the recipient after recognition of the antigens on the transplanted tissue, and the degree of response varies depending on the organ/tissue transplant. The grafts placed in sites that do not possess lymphatic drainage or vasculature, are not rejected. The examples include the brain, testes, and anterior chamber of the eye, which are also called immunologically privileged sites. However, the skin, which does not possess major blood vessels at the time of the graft, later acquires the vessels, lymphocytes of the recipients enter the graft, and its antigens are transported back to the lymph nodes, resulting in the development of an effector response.

The final phase in graft rejection is termed as the efferent phase or effector phase. After activation in the spleen or lymph nodes, T cells, B cells, and monocytes enter the transplanted tissue. The nature of the effector response corresponds with histoincompatibility and whether the rejection involves a primary or secondary graft. The most common effector mechanisms involved in tissue rejection include delayed-type hypersensitivity and cytotoxic T-cell-mediated lysis. The other mechanisms include antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, which are less frequent. Histological analysis of rejected tissue has demonstrated the infiltration of $CD4^+$ T cells and macrophages in the graft, and the infiltration is similar to that observed in delayed-type hypersensitivity reaction. T cells cause tissue damage by the elaboration of cytokines, such as IFN-γ, TNF-α, and TNF-β, and also by HLA Class II-restricted cell-mediated cytotoxicity. Cytokines such as IFN-γ, TNF-α, and TNF-β have direct as well as indirect effects in this process. An example of the indirect effect is the augmentation of the expression of HLA molecules by these cytokines. IL-2 is required for T-cell proliferation, and the production of $CD8^+$ T cells and the local production of inflammatory cytokines and IFN-γ increase HLA expression in the graft. The recipients' $CD8^+$ T cells play a dominant role in the killing of the graft. During rejection of the kidney, liver, heart, and pancreas transplants, the levels of HLA Class I and HLA Class II molecules increase dramatically. The HLA Class I molecule expression is increased in all grafted tissues including the endocrine cells of the pancreas; however, the induction of the HLA Class II molecules is more selectively induced as is the case with hepatocytes where there is no induction of HLA Class II molecules.

When compared to allorecognition by T cells, the role of MHC molecules is very different in allorecognition by NK cells. On NK cells the absence of self-MHC Class I molecules causes activation, and there is a protective effect of self-MHC Class I molecules against killing by NK cells. As a result MHC-mismatched cells are a target of NK cells, and inhibition of T-cell response may not result in the desired outcome of immunosuppression.

Lifelong immunosuppressive therapy is required for the solid organ transplantation because tolerance in the host does not develop for the donor tissue. But in allogeneic hematopoietic transplantation, the host eventually is populated by the hematopoietic cells of the donor. When the probability of developing graft-versus-host disease subsides and other rejection manifestations are treated following allogeneic hematopoietic transplantation, an immune response is generated, which is tolerant to both the host and the donor. Both central and peripheral tolerance mechanisms are involved. In case of a successful allogeneic hematopoietic transplant, only 1 year after treatment with the immunosuppressive drugs, they are no longer needed.

Adhesion molecules also play a crucial role in allograft rejection. The migration of activated T cells from secondary lymphoid organs to the graft site is regulated by adhesion molecules. Furthermore, these molecules are pivotal in the interaction of T cells with the functional components of tissue allografts that are epithelial in origin.

8.4 Migration of Effector or Memory Cells

The rejection of allograft depends on the migration of effector or memory T cells to the graft. It is believed that induction of Gα_i-coupled chemokine receptors on T cells regulates crucial steps involved in the migration of T cells. This involves integrin-dependent adhesion, resulting in transendothelial migration. It has also been suggested that the firm adhesion and transendothelial migration of graft antigen-specific effector cytolytic T cells require the presence of cognate antigen. Furthermore, this process is independent of Gα_i signaling. In this case, T-cell migration only requires cognate antigen presentation by antigen-presenting cells, derived from the bone marrow or graft endothelial cells. However, the antigen-nonspecific effector T cells are dependent on Gα_i signaling for the adhesion and transmigration, and the presence of allograft-specific effector T cells is required. This is another pathway for the migration of effector T cells into vascularized tissue transplant. These later findings raise the questions about the role of cognate antigens, during their presentation and resulting migration of T cells into the graft for the rejection.

Memory T cells specific for allograft antigens present a serious challenge in organ transplantation. The presence of memory T cells not only results in a robust immune response to a transplanted organ but may also be responsible for a poor response to the immunosuppressive drugs. Lymphoid sequestration of memory CD4$^+$ T cells prolongs survival of allografts in animal models, which can be achieved by immunosuppressive drugs such as fingolimod which influences memory CD4 T-cell trafficking.

8.5 Role of Innate Immune Response in Allotransplant Rejection

The role of acquired immune response alone in allograft rejection does not provide the complete scenario of the interplay of events associated with rejection. Before the induction of T-cell response, there is production of pro-inflammatory mediators, which is the result of the innate immune response. The innate immunity recognizes nonself-molecules, which are unique to the invading pathogens. Such recognition produces a robust inflammatory response based on the presentation of antigen and results in the proliferation and differentiation of myeloid cells. Innate immune system utilizes nonrearranged receptors called pattern recognition receptors (PRRs). PRRs identify foreign self from nonself. Toll-like receptors (TLR) defined in Chap. 1 are a family of PRRs. They are expressed on a variety of tissues including dendritic cells, T cells, B cells, mast cells, organ parenchyma cells, and endothelial cells. The inflammatory mediators and systemic danger signals regulate the expression of TLRs. One of their roles is to keep an eye on the extracellular environment through their ligand-binding regions. The detection of nonself will result in a series

of events, including the induction of transcription factors NF-κ and AP-1. As a consequence, TLR involved the production of pro-inflammatory cytokines, chemokines, and adhesion molecules. They also play a role in augmented antigen presentation via antigen-presenting cells and induction of costimulatory molecules.

NOD-like receptors (NLRs) and the RIG-like helicases (RLHs) are soluble proteins, which are part of the PRR families and are involved in the detection or surveying of intracellular compartments. NOD and NALP are subclasses of NLRs. NALP3, a subclass of NALP family, in association with two adopter proteins forms a structure called NALP3-inflammasome, after the previous complex associates with capase-1. Inflammasomes are involved in controlling the induction of IL-1β and IL-18. RAGE, the receptor for advanced glycation end products, is a transmembrane receptor of immunoglobulin super family and possesses an inflammatory function in innate immune response. Binding of RAGE to its ligand causes pro-inflammatory gene activation. RAGE is also involved in leukocyte involvement via being a receptor for β_2 integrins.

The acquired immune response is induced by the antigen-presenting cells that uptake, process, and present the antigenic fragments of the microbial agents to the T cells, consequently producing a thymus-dependent immune response against the pathogen. Although allografts are devoid of such microbial pathogen molecules, nonetheless, they produce a strong tissue rejection response. This may be the result of continued differentiation of host monocytes into mature dendritic cells. These dendritic cells release IL-12, induce proliferation of T cells, and produce IFN-ϒ. In case of syngeneic graft, there is attenuated development of dendritic cells from monocytes and a lack of production of IL-12 and IFN-ϒ. This suggests that while there is a need for the recognition of allogeneic nonself by the innate immune system, the danger signal alone is not sufficient and requires the detection of allogeneic nonself by the innate immune response. The danger signal constitutes the release of the "danger" molecules from the dying cells in the graft. It has been hypothesized that the "danger" molecules released by the dying cells in the allograft cause the maturation of antigen-presenting cells and induce the development of acquired alloimmune response. These observations link innate immune response to acquired immune response via induction and maturation of dendritic cells as the mechanism of allograft and perhaps autologous graft rejections. The allograft rejection of donor-reactive T cells is mediated via multiple mechanisms. In one mechanism there is synergism between graft-infiltrating neutrophils and donor-reactive CD8+ memory T cells, which increases intragraft inflammation. This results in the recruitment of effector T cells to the graft, which are donor antigen primed.

8.6 Types of Organ Rejection

The rejection period for a graft varies from tissue to tissue and the type of immune response involved in this process. They are divided into three types: hyperacute rejection, acute rejection, and chronic rejection.

8.6.1 Hyperacute Rejection

This is a rare type of rejection that can occur very rapidly within a few days, which is the result of pre-existing antibodies to the donor antigens. The events include a big infiltration of neutrophils into the grafted tissue caused by antigen-antibody complexes that activate the complement system and major clotting within capillaries as a result of inflammatory reactions, which does not permit the vascularization of the graft. Most grafts in clinical transplantation are vascularized and are directly linked to the host's circulation. In hyperacute rejection, these pre-existing antibodies react with antigens on the vascular endothelial cells and cause immediate death of the graft. The cytotoxic antibodies against HLA antigens are formed due to several factors, which include repeated blood transfusions, several pregnancies where repeated exposure to paternal alloantigens of fetus results in the development of antibodies to these antigens, and/or a previous transplant where antibodies are still left against the antigens of the original graft. At the present time, all recipients are screened for the presence of antibodies to the donor antigens, thus minimizing the chances of hyperacute rejection.

8.6.2 Acute Rejection

About 10 days after transplantation, acute rejection of the graft begins as a result of cell-mediated immunity. Acute rejection is a result of infiltration of large numbers of macrophages and lymphocytes into the graft. Helper T-cell activation and proliferation play a major role in this process, and both the complement- and antibody-dependent cell-mediated cytotoxicity are involved in the destruction of the graft. Acute rejection could be in the form of acute vascular rejection, acute cellular rejection, or both. Acute vascular rejection involves the necrosis of the blood vessel cells of the graft where thrombotic occlusion is not observed, but histologically the pattern is similar to vasculitis. This form of rejection is mediated by IgG antibodies that are directed against the alloantigens of endothelial cells and involve complement activation. T cells and cytokines, which induce endothelial necrosis, also contribute to acute vascular rejection.

Acute cellular rejection involves the infiltration of macrophages and lymphocytes into the graft and is evident by the necrosis of the parenchymal cells of the graft. The lysis of the parenchymal cells of the transplanted tissue is achieved by the infiltrating leukocytes. Acute cellular rejection may be the product of several mechanisms including cytolytic T-cell-medicated lysis, natural killer cell-mediated lysis, and activated-macrophage-mediated lysis. The acute cellular rejection predominantly involves CD8+ T-cytolytic cells that kill the grafted tissue.

8.6.3 Chronic Rejection

In some cases, a slow rejection phase begins many months or even years after transplantation when acute rejection has subsided. The chronic rejection appears to be due to the slow buildup of antibodies against the graft antigens and/or due to

cell-mediated immune responses by the recipient. It does not respond well to the immunosuppressive drugs, and a new transplant is needed following chronic rejection reaction.

Fibrosis resulting in the loss of normal organ structures is the hallmark of chronic rejection. The fibrosis may be due to wound healing, which is then followed by the cellular necrosis of acute rejection. However, it must be pointed out that chronic rejection develops many times in the absence of acute rejection. Fibrosis may be a result of several diverse factors such as equation of chronic rejection with chronic delayed-type hypersensitivity reaction, injury to blood vessels and resulting response to chronic ischemia, the proliferation of smooth muscle cells in the intima of arterial walls producing vascular occlusion, or persistent viral infections that will induce cellular immune response.

8.7 Transplant Tolerance

The long-term acceptance of an allograft is the basic purpose of tissue transplantation. However, the mechanisms potentially involved in achieving this goal are unknown. A number of hypotheses have been put forward to describe the mechanisms favoring tolerance to an allograft. According to one, in long-term survivors of tissue transplantation, there is clonal deletion of lymphocytes specific for the alloantigens of the graft, and in these recipients, the T cells responsible for attacking and killing the graft have been specifically eliminated. The second hypothesis suggests that T cells specific for the alloantigens of the graft have become anergic and consequently they lose their effector function and are unable to attack the graft in the recipient. The third suggested mechanism is the development of regulatory T cells in transplant tolerance. The concept of suppressor T cells in achieving transplantation tolerance was introduced many years ago; however, their definite role was allusive since no markers were identified to characterize these cells and delineate their role in tissue transplantation and immune tolerance. In 1990, Hall and colleagues reported that a subset of T cells ($CD4^+$) which also expressed CD25 marker transferred tolerance in a specific manner. Later Sakaguchi et al. reported that $CD4^+$ $CD25^+$ subset of T cells possessed suppressive properties and consequently the term suppressor T cells was replaced by regulatory T cells. The interaction of tolerogenic dendritic cells with regulatory T cells may downregulate the host's response to an allograft resulting in long-term graft survival. The tolerance induction may be achieved by blocking various cell surface markers including CD4, CD8, CD28, CD40L, CD80, and CD86 and as a result of the proliferation of regulatory T cells. One concept understudy is an ex vivo expansion and administration of graft-specific regulatory T cells to the recipients of tissue transplantation. Nonetheless, T-cell regulation to achieve allograft tolerance is a difficult task since allografts are always rejected without immunosuppression.

8.8 Procedures of Preventing the Graft Rejection

According to the laws of tissue transplantation, an allograft will be rejected. Since a good match between the graft and the recipient aids in prolonged acceptance of the transplanted tissue, one strategy of significance is to minimize the immunogenicity of the graft. The matching of the three or four alleles of four HLA-A and HLA-B loci is preferable. HLA compatibility is also assessed for both HLA Class I and HLA Class II molecules. During rejection, the immune response results in the destruction of the transplanted tissue. The CD95 (Fas or Apo-1) ligand (FasL) in the allograft is responsible for counteracting rejection by causing the apoptosis of lymphocytes and monocytes carrying the Fas and may induce an immune privileged status in allografts. Although this approach is still under investigation, other approaches have been used in clinical practice to improve the potential of transplant acceptance. Immune suppression before the transplantation of the graft is a major practice to avoid or delay the rejection process. The focus has been to pharmacologically eliminate or reduce T cells, which is achieved by the use of the immunosuppressive drugs or irradiation to suppress the immune response of the recipient. The pre-existing antibodies are removed by plasmapheresis, and drugs including rapamycin, 15-deoxyspergualin, and brequinar have been used to inhibit antibody synthesis. Other strategies include the attempts to induce tolerance in the host. Renal transplant patients who were given multiple blood transfusions accepted kidney transplants better than their counterparts who did not undergo multiple allogenic blood transfusions. Consequently, in clinical practice multiple transfusions of blood have been used to induce tolerance prior to transplantation. Other strategies under trials include the use of peptides obtained from grafts' HLA molecule and administering the recipient with their high doses to induce tolerance. HLA donor molecules, and the antagonism of costimulatory molecules so the transduction signal to T cells is blocked, when they recognize antigens of the graft, thus inducing T-cell anergy, have also been employed. The use of soluble CTLA-4 to antagonize the signal transduction between B7 molecule of the graft and $CD28^{+}$ T cell of the recipient is also of interest since this inhibits the production of IL-2, which induces anergy in graft-activated T cells.

8.9 Clinical Issues in Tissue Transplantation

The availability of the tissue or organ is the first factor for tissue transplantation. United Network for Organ Sharing (UNOS) is a program designed for the collection and delivery of donor tissues or organs nationwide. The shortage of organs for transplantation, along with the viability of the tissue or organs, continues to be a problem. A graft is either a vascularized or nonvascularized organ, and in cases of vascular organs, which include the heart, lung, liver, and kidney transplants, surgical anastomosis of the vascular system of the recipient is required. In contrast, the

nonvascularized organs include islets of Langerhans, and they are transplanted in a tissue where the presence of sufficient tissue fluids allows the delivery of the nutrients and the removal of the waste products. The survival of this tissue is not dependent on its attachment to the vasculature, and its normal function is expected. The successful harvesting of any tissue or organ is important for the desired outcome of tissue transplantation such that there is a minimum time of ischemia for each tissue.

Before a tissue is considered for grafting, it is important that it should be matched with the recipient for HLA-A and HLA-B alleles. The graft survival improves with the degree of matching of HLA-A and HLA-B alleles between the donor and the recipient. This matching plays a major role in avoiding a major rejection during the first year after the tissue transplant. Since HLA-C does not play an important role in T-cell recognition, its alleles are not generally matched.

The tissue typing is done to determine the compatibility of the graft and the recipient for HLA and blood groups, and the existence of pre-existing antibodies against the grafted donor tissue is also detected by using complement-dependent lymphocytotoxicity assay. A positive crossmatch will suggest a risk for hyperacute rejection and is indicative of a higher risk for vascular rejection immediately after transplantation, and consequently the transplantation of this tissue is contraindicated. This problem is not usually observed for liver transplants but is important for heart and kidney transplants. The crossmatch assays are modified for kidney transplantation to increase their sensitivity. The techniques used include ELISA, flow cytometry, B lymphocytes crossmatches, and antihuman globulin augmentation. The presence of alloreactive antibodies resulting from pregnancies, blood transfusions, and previous transplants is determined in the serum by testing against a random cell panel. The results are expressed as percentage panel reactive antibody, which reflects the degree of sensitization and can vary from non-sensitivity (0 %) to high sensitivity (80–100 %). The task for finding a crossmatch negative donor decreases as the degree of sensitivity increases. The serum screening procedure is devised to detect HLA-specific antibodies. HLA molecules that have multiple epitopes are divided into two groups, private and public determinants. The examples of private determinants include HLA-A and HLA-B7. The public determinants, also called cross-reactive groups, are antigenic determinants where molecules with different private specificities share an epitope and include $A2^{+}A9^{+}A28^{+}$ and $B7^{+}B22^{+}B27^{+}B40^{+}$. The highly sensitive patients exhibit the same pattern of reactivity against a single or multiple public antigenic determinants. Cellular rejection is mediated by T lymphocytes.

In addition to matching HLA-A and HLA-B, matching of HLA-DR alleles is important. Due to strong linkage disequilibrium between HLA-DR and HLA-DQ, these two loci often match but DP typing is not done. As expected, due to a diverse population, the matching is often difficult in the United States as opposed to the countries where there is more inbreeding. The graft's acceptance is also dependent on the HLA-DR type of the recipient. This is independent of matching at HLA-A, HLA-B alleles. This phenomenon is due to "immune response (IR)" gene effect, because mature T-cell repertoire selection is dependent on the host's HLA-DR molecules. An immune response to a graft antigen will not develop if the host's T cells lack specificity for that alloantigen.

8.10 Graft-Versus-Host Disease

Billingham in 1966 described the development of graft-versus-host disease as a result of the presence of immunocompetent cells in the graft, foreignness of the graft, to the recipient, and the inability of the recipient to mount an effective immune response against the graft. This disease results principally from bone marrow transplantation because the bone marrow contains the pluripotent hematopoietic stem cells, and the presence of immunocompetent cells in the graft allows it to mount an immune response against the recipient, which is called graft-versus-host reaction. This injures the host, and the process is called graft-versus-host disease.

The injury to the immune system of the host results in the graft-versus-host reaction, which either could be acute or chronic, that is, a response as the recipient tries to prevent the rejection of its stem cells. Epithelial cell necrosis characterizes the acute graft-versus-host disease, which mainly affects three organs, the liver, gastrointestinal tract, and skin. Skin rash, diarrhea, and jaundice are the clinical manifestation of acute graft-versus-host disease. Acute graft-versus-host disease may result in death if the epithelial lining of the gastrointestinal tract is completely damaged or the necrosis of skin is major. In chronic graft-versus-host disease, there is no acute cell necrosis but atrophy and fibrosis of the liver, gastrointestinal tract, and/or skin take place. The symptoms of chronic graft-versus-host disease also include skin rash, liver abnormalities, inflammation of the skin, mouth lesions, hair loss, indigestion, lung damage, and drying of the eyes and mouth. Both acute and chronic graft-versus-host disease increase the risk of infections, and death will result from the organ failure. The acute graft-versus-host disease is manifested within 8 weeks of the transplant, whereas the chronic form is seen within 12 weeks. The removal of the mature T cells from the bone marrow reduces the development of graft-versus-host disease, but it also makes the transplantation less effective. The colony-stimulating factors released by mature T cells assist in the homing of the stem cells (Fig. 8.2).

It has been suggested that single nucleotide polymorphisms in various cytokines may determine the severity of graft-versus-host disease, and these polymorphisms may suppress or enhance the inflammatory responses of the recipient. A role of IL-10 and not of IL-1β, IL-6, and TNF-α is suggested in the outcomes of graft-versus-host disease. It appears that factors other than histocompatibility between the graft and the recipient and the residual number of T cells in the graft may also be responsible for the pathogenesis of graft-versus-host disease. For example, the development of graft-versus-host disease in the recipients of either syngeneic or autologous bone marrow transplants cannot be explained only on the basis of major and minor histocompatibility differences, and the disease develops even when patients are taking cyclosporine. The mechanisms of cyclosporine-induced graft-versus-host disease in autologous bone marrow transplantation may be a result of dysregulation of self-tolerance.

In graft-versus-host disease, immunocompetent cells recognize epithelial lining of the target tissue as foreign, which induces an inflammatory response followed by the death of the target tissue by apoptosis. The apoptosis is not solely dependent on

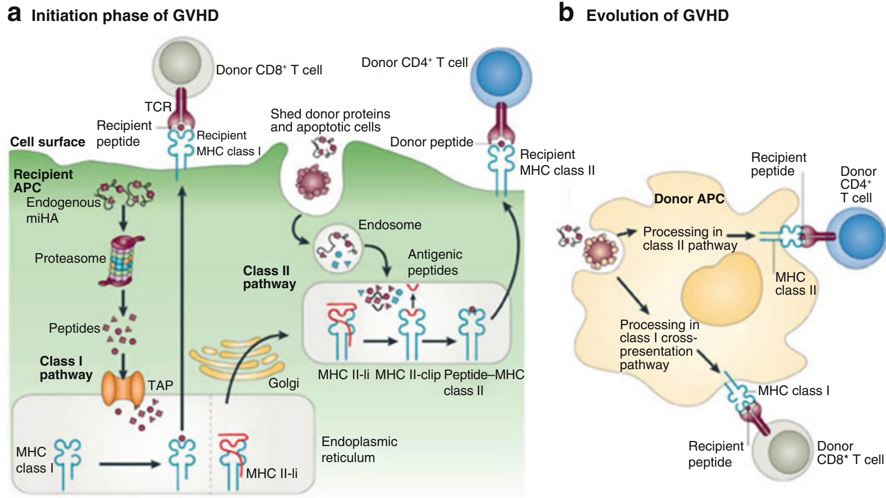

Fig. 8.2 Mechanism of graft-versus-host disease: (**a**) in the first step, cytolytic T cells of the donor recognize peptide fragments of minor histocompatibility agents. In the antigen-presenting cells of recipient, polypeptides are broken down and are transported to endoplasmic reticulum. That is where they are associated with MHC Class I molecules. The peptide fragments in context of MHC Class I molecules on the cell surface of antigen-presenting cells are then recognized by cytolytic T cells of the donor as nonself. However, the activation of helper T cells is in response to antigens that are processed by the antigen-presenting cells of either the recipient or donor (**b**). This processing is through the Class II pathway. The helper T-cell-mediated graft-versus-host disease could result from recognition of only foreign non-hematopoietic antigens. Furthermore, helper T cells of the donor can recognize antigens which are synthesized endogenously by antigen-presenting cells or obtained exogenously from the hematopoietic cells of other recipients. This is followed by activation of donor cytolytic T cells by donor antigen-presenting cells. Nonself antigens are cross-presented in this case (Reproduced with permission. Source: Shlomchik WD (2007) Graft vs. host disease. *Nature Review Immunology*. 7: 340–352. Nature Publishing Group)

alloreactive T cells derived from the nonidentical donor, as it will take place even if the alloreactive T cells are from the recipient. While T cells are responsible for the development of the initial inflammatory response, other cell types including natural killer cells are present on the site of epithelial injury and play a role in graft-versus-host disease.

8.11 Clinical Transplantation

8.11.1 Kidney Transplants

The first kidney transplants were done in the 1950s. In the cases of advanced and permanent kidney failures, kidney transplantation is a potential treatment option where a kidney donor could be any normal healthy adult with normal kidney

function. The absolute contraindications include inability to successfully administer the anesthesia or the immunosuppressive therapy to the recipient and the presence of disease such as cancer, cardiopulmonary problems, infection, and/or untreated peptic ulcer. The immunologic evaluation performed prior to the grafting includes blood grouping, HLA testing, presence of pre-existing antibodies and/or T cells to HLA antigens, and detection of viral infections. A kidney from a blood group A can be transplanted to recipient with blood group A or AB; for donor blood group B, the recipient should be of the blood group B or AB; for donor blood group AB, the recipient must have the same blood type; individuals with blood group O are universal donors. The blood grouping is followed by HLA testing to evaluate the compatibility. The siblings (brothers and sisters) present the optimal chance for an excellent match (six of six HLA). However, kidneys from unrelated donors are also used because of advances in immunosuppressive therapy. Crossmatching is done to determine the presence of pre-existing antibodies; a negative crossmatch results in the acceptance and a positive crossmatch is rejected by the recipient. The potential donor is also evaluated for cancer, diabetes, renal function, heart disease, and/or infection. In addition to the blood tests for renal function, a three-dimensional CAT scan of the kidney and a renal angiogram is performed to exclude any potential abnormalities in kidney's physical condition and the kidney's blood vessels. Furthermore, preoperative angiogram shows if the right or left kidney will be selected for grafting. If a potential donor is not available, cadaveric transplantation is considered. In most cases, the failing kidneys are not removed but the graft is placed generally in iliac fossa and a different blood supply is used. The renal artery of the donor kidney is generally connected to the external iliac artery of the recipient. The renal vein of the donor kidney is generally linked to the external iliac vein of the recipient. The surgery takes about 3 h. The new kidney is expected to function immediately after surgery but may take up to a few days.

The long-term outcome of the transplant is dependent on the extent of the matching. The success rate decreases as the number of matching antigens decrease from six to five to four and so on. Nonetheless, due to the availability of the immunosuppressive drugs, mismatched grafts, that is, zero, antigen-matched organ, also do well, and consequently, good tissue matching is preferred but not absolutely required for the successful outcome of kidney transplantation.

Postoperative immunosuppressive therapy is the most important aspect of the procedure. The most common immunosuppressive regimens include tacrolimus, mycophenolate mofetil, and prednisone. Other regimens include cyclosporine, rapamycin, azathioprine, or monoclonal antibodies. Hyperacute rejection is no longer a problem due to crossmatching before the transplant. Acute rejection will be exhibited in 10–25 % of the patients within 60 days of grafting. This may not be an indication of total failure, but rather reflective of a need for adjusting the immunosuppressive therapy. The symptoms of acute rejection include swelling and tenderness over the graft and a decrease in kidney function. The transplant rejection could also be chronic. Kidney transplantation is a life-extending surgery as it prolongs life from 10 to 15 years on average. As expected, the young benefits more than the elder recipients where life is extended up to 4 years. It is recommended that the kidney

transplant should be preemptive since extended periods of dialysis negate the period of survival of the grafted kidney.

8.11.2 Liver Transplantation

The liver transplantation was first performed in 1963 in the United States and United Kingdom. However, the success was not achieved until 1967 when a recipient survived 1 year after the surgery. It remained an experimental procedure until the 1980s, which also coincided with the introduction of cyclosporine. In the 1970s, patient survival for 1 year was 25 %, which has now improved to 85–90 %. The indication for liver transplantation is an acute or chronic condition which causes an irreversible dysfunction of the organ with possible death within 2 years. The contraindications for the procedure include the recipient's HIV seropositivity, extrahepatic malignancy, hepatitis B seropositivity, drug and alcohol use, heart and lung disease, advanced age, and/or active septic infection. However, exceptions to HIV seropositivity are now considered. ABO blood matching is done to select a suitable donor, and organ sizing is used for the suitability of the graft. The approach of organ sizing has been changed since now livers can be made smaller by surgery, which is particularly useful for transplantation in infants and children when an adult liver is used. Based on the regenerative capacity of the human liver, living-donor liver transplantation has become a new option in recent decades. This procedure was originally performed in Brazil in 1986 for a child, but it was eventually realized that adult to adult transplantation was also feasible. The transplantation procedure is orthotopic as the damaged liver is removed and the donor liver is placed in the same location. There are three phases of the surgery: (a) hepatectomy, (b) anhepatic phase, and (c) postgrafting phase. The hepatectomy involves severing all attachments of ligaments, bile duct, portal vein, and hepatic artery; the implanting of the graft involves connecting the inferior vena cava, portal vein, and hepatic artery, which is followed by connecting the bile ducts after the blood flow to the grafted liver is restored. On average, the surgery takes 4–7 h.

Human leukocyte antigen typing and leukocyte crossmatching are not done due to relatively short maximum cold ischemic time. ABO-compatible matches are also used under certain conditions since ABO identical grafts have better survival rate than compatible nonidentical grafts. Hemolysis is a major problem in ABO nonidentical liver transplants, which results from graft-versus-host reaction, which is due to the production of antibodies by the donor lymphocytes against ABO antigens of the recipient. Hemolysis is observed between 4 and 7 days after the transplantation. The symptoms include fever, increases in serum bilirubin levels and reticulocyte count, and a decrease in serum hemoglobin levels. Hemolysis can be addressed by hydration, diuretics, blood transfusion, and plasmapheresis. HLA typing is also not performed because the liver is not very antigenic. Although expression of HLA Class I molecules is minimal on hepatocytes, the presence of Kupffer cells, vascular

endothelial cells, and other inflammatory cells may cause rejection of the graft; therefore, rejection is carefully monitored.

There are only a few reports of hyperacute rejection after liver transplantation. This may be due to the ability of the Kupffer cells to remove cytotoxic antibodies formed against the graft because of their reticuloendothelial function. Acute rejection is the more common form of rejection that is manifested within 7–10 days after liver transplantation and exhibits symptoms of fever, malaise, pain, tachycardia, and hepatomegaly. Mental disorientation in patients has also been reported during acute rejection. Liver biopsy is performed to confirm acute rejection that is generally mild in nature, and lymphocytic infiltration is observed in the portal tracts under the endothelium of the sinusoids.

Chronic rejection after liver transplant is rare (<3 %), which is attributed to the recognition of acute rejection and the ability of the liver to regenerate itself. During chronic rejection, there is progressive failure of liver function, and humoral immune response is involved in this process as the liver exhibits fibrosis, arteriolar thickening, and loss of bile ductules resulting in the loss of liver function, portal hypertension, and jaundice. Chronic rejection can now be treated with immunosuppressive therapy and a new transplant is not needed. The immunosuppressive drugs for liver transplantation include corticosteroids in combination with tacrolimus, cyclosporine, or sirolimus. The risk of chronic rejection decreases over time but the immunosuppressants are taken on a permanent basis. This may be attributed to genotypic chimerism in the bone marrow of patients undergoing liver transplantation. The most critical period is within 3 months after the transplantation, and the 5-year survival rate is 78 %.

8.11.3 Pancreas Transplantation

The pancreas was first transplanted in 1966. This was a transplant of multiple organs where kidney and duodenum were also transplanted in a 28-year-old woman; she exhibited a decrease in sugar levels immediately after transplantation but died 3 months later due to pulmonary embolism. The first partial pancreatic transplant, in which the donor was a living relative, was performed in 1979, but until 1990, it was considered as an experimental procedure.

The transplantation of the pancreas is mostly performed in individuals with type I diabetes and is life-enhancing and not a lifesaving procedure. The indications for the transplant include diabetes, neuropathy, nephropathy, or retinopathy. In addition to transplantation of the pancreas by itself, the procedure could also be simultaneously performed as pancreas–kidney transplantation or pancreas transplantation after kidney transplantation. The simultaneous pancreas–kidney transplantation is performed when both organs are from the same deceased person; the majority of pancreas transplantation (>90 %) are simultaneous pancreas–kidney transplantation. The pancreatic transplantations could be performed with either as a whole

organ, a segment, or islets of Langerhans. Ideally, the transplant should be performed before the development of any diabetic complications. The pancreas transplant donors and recipients are first matched for blood group compatibility. The details of the requirements have already been described in reference to the kidney transplant. Rh factor is not considered for blood typing. The HLA testing is done for six markers including Class I HLA-A, HLA-B, and HLA-C and Class II HLA-DP, HLA-DQ, and HLA-DR as the number of proteins that these six markers encode is between 10,000 and 13,800. The panel reactive antibody test is also performed to rule out the presence of pre-existing antibodies.

The candidates for pancreatic transplantation are evaluated for renal disease, diabetic retinopathy, coronary artery disease, gastroparesis, coronary artery disease, stroke, autonomic neuropathy, and peripheral vascular disease. Gastroparesis (deficient gastric emptying) affects the use of immunosuppressive agents after transplantation, and other drugs including cisapride or metoclopramide are administered to address the problem. Each candidate is thoroughly tested clinically before determining the suitability of the procedure. The tests include blood chemistry, CBC count, liver function test, kidney function, chest radiography, exercise scintigraphy, and, in some cases, stress cardiac ultrasound and/or cardiac arteriography. One of the most important tests is assessing the potential recipient for an existing infection, which may include hepatitis B and C, HIV, cytomegalovirus, tuberculosis, and Epstein–Barr virus.

There are many different procedures that are used for pancreatic transplantation and there is no one standard protocol used in all transplant centers. The important considerations, however, are that the arterial blood flow supply to the pancreas and duodenal segment, and venous outflow from the pancreas via the portal vein should be adequate. The recipient's right common or external iliac artery is used to restore vascularization of the artery in the pancreas. The Y graft of the tissue is anastomosed end to side and the venous vascularization is performed either systemically or portally, but mostly it is done with systemic venous drainage.

Desired immunosuppression for pancreas transplantation is more challenging than kidney or liver transplants. T-cell alloimmune rejection response is a major problem, and lifelong immunosuppressive therapy must be provided. A short course of immunosuppressive therapy using intravenous treatment with antilymphocytic polyclonal antibodies or monoclonal antibodies, including muromonab, daclizumab, basiliximab, or alemtuzumab, is helpful, which provides protection from early acute rejection. Specifically, the antibody treatment is important when other immunosuppressive agents such as cyclosporine, tacrolimus, or sirolimus are either not administered or sub-therapeutic doses are used until the function of the grafted pancreas improves. The maintenance immunosuppressive agents include prednisone, azathioprine, cyclosporine, tacrolimus, or sirolimus. The current survival rate for pancreas transplantation is 97.6 % after 1 year, the functional survival rate after 1 year of pancreas transplant is 85 %, and the success of a transplant is dependent on the age of the donor and HLA match.

8.11.4 Heart Transplantation

Ancient mythology and biblical references mention heart transplantation, but after the initial work of Alexis Carrel in the early 1900s, dog heart transplants were performed at Mayo Clinic in 1933. The work of Norman Shumway at Stanford University, reported in 1959, led to the first human transplant by Christian Bernard in 1967 in South Africa. By the 1970s the enthusiasm diminished due to poor survival rate after heart transplantation until the discovery of cyclosporine.

Today, the indication for cardiac transplantation is the presence of end-stage heart failure or severe coronary artery disease. In general, normal functioning heart is removed from a recently deceased donor and is placed in a recipient patient. The recipient's original heart is taken out mostly by an orthotopic procedure. Although xenografts and artificial hearts have also been used for transplantation, they have exhibited limited success. Cardiac donors are heart beating, brain-dead cadavers without any history of myocardial disease. The recipients have advance irreversible heart disease, which may include congenital heart disease, heart valve disease, cardiomyopathy, life-threatening arrhythmias, and/or coronary artery disease. The recipient could be less suitable if other circulatory disease is present. If cardiac function is normal, advanced donor age is no longer a contraindication. Donors and recipients are tested for ABO blood group compatibility and are matched for heart size. The recipients are also screened for the presence of pre-existing HLA antibodies. For heart transplants, HLA matching is not routinely done despite concern from some cardiologists. One limiting factor is the period of viability of the heart, which does not allow enough time for HLA matching. Increased compatibility improves outcome despite suggestions that the availability of an excellent immunosuppressive regimen will result only in a slight improvement in the outcome if HLA matching is done.

A heart is transplanted in a heterotopic or orthotopic position. Heterotopic transplant is preferred in patients with severe pulmonary hypertension. Orthotopic transplantation is done according to the technique developed by Shumway and Lower, or as a bicaval anastomosis. Immunosuppressive therapy is initiated right after the transplant surgery. There are several immunosuppressive regimens, which include both pretransplantation induction therapy and posttransplant maintenance therapy. Each transplant center has its own preference for the choice of immunosuppressive drugs, which include cyclosporine, tacrolimus, sirolimus, and/or corticosteroids. Hyperacute rejection has been observed only rarely in allograft cardiac transplants but could occur immediately after restoring the blood flow for about a week. During the first month after transplantation, endomyocardial biopsies are performed weekly to detect rejection, and the frequency diminishes with time. Acute rejection is expected and the incidence diminishes after 6–12 months of transplantation but could take place at any time after that as well. Treatment of acute rejection includes corticosteroid and polyclonal or monoclonal antibodies depending on the severity of the rejection. Lymphocytes, lymphoblasts, and monocytes are the predominant

cells in acute rejection. Late deaths, 1 year after transplant, are attributed to chronic rejection, which may also involve humoral mechanisms. Currently, the average patient and graft survival rate after 1 year is 85–90 % and 3-year survival is 75 %. The median survival after heart transplantation is 11 years.

8.11.5 Lung Transplantation

The first human lung transplant was performed in 1963. Today, it is considered an excellent alternative for children with end-stage lung disease. Single-lung, double-lung, and living-donor lobar lung transplantation have been performed. The lung transplantation is recommended in patients with untreatable end-stage disease attributed to multiple etiologies. Congenital heart disease, pulmonary vascular disease, and/or idiopathic pulmonary hypertension result in end-stage lung disease in children less than 1 year old. Cystic fibrosis results in end-stage lung disease in children who are 1–10 years old and is responsible for 36 % of the total lung transplants. Pulmonary fibrosis, congenital cardiac disease, and chronic lung disease of infancy also commonly lead to lung transplants. Donors and recipients are matched for size, thoracic dimensions, and ABO blood group. Crossmatching is also done to exclude the possibility of the presence of pre-existing antibodies. Absolute contraindications include the presence of malignancy, tuberculosis, infection, neuromuscular disease, renal malfunction, and/or immunodeficiency disorders. The potential donors are screened for infections and smoking habits and should be less than 60 years old. The pediatric surgical technique most commonly used in the United States is bilateral sequential procedure with telescoping anastomoses. Lower lobe of the lung is used if a living lobar family donor is used, and this is no longer an emergency procedure, but rather an elective one. Immunosuppression after the lung transplantation is achieved by using cyclosporine, tacrolimus, sirolimus, everolimus, corticosteroids, IL-2 receptor antagonists, and/or azathioprine. Acute pulmonary rejection is treated with short-time increased corticosteroid therapy or by using polyclonal or monoclonal antibodies including anti-lymphocytic immunoglobulins or OKT3. The symptoms of rejection include flu-like symptoms, fever, breathing difficulties, nausea, chest pain, and decreased pulmonary function. Chronic rejection is manifested in about 50 % of the patients and is presented as bronchiolitis obliterans. Survival rates are 78 %, 65 %, and 51 % at 1, 3, and 5 years, respectively.

The first combined heart–lung transplant was performed in 1981 at Stanford University. Combined heart–lung donors need to satisfy both the requirements already described separately. Combined heart–lung transplant is recommended in patients with congenital problems affecting these organs, pulmonary hypertension, and/or cystic fibrosis. The recipients for the combined transplant are recommended to be less than 55 years old. Survival rates are about 84 %, 66 %, and 54 %, for 1 year, 3 years, and 5 years after transplantation, respectively.

8.11.6 Bone Marrow Transplantation

Bone marrow is used to treat both malignant and nonmalignant diseases. The foundation for the bone marrow transplantation was set by E. Donnell Thomas between the 1950s and 1970s at the Fred Hutchinson Cancer Research Center, and the first successful allogeneic bone marrow transplant was performed in 1968. The patient's bone marrow is either damaged by high doses of chemotherapy or is subject to irradiation due to malignancy. Transplanting autologous bone marrow comes with a lower risk of graft-versus-host disease, infection, and rejection.

The most compatible matches come from fully matched family members. Routine testing involves matching of six antigens, HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ, and HLA-DP, each of which has two alleles, and the recipient and the donor are considered a mismatch if all six antigens are different. The haplotype donors match only three of the six antigens. It is important to match all six antigens when the donor is not related to the recipient. The transplantation procedure is divided into five phases, which include conditioning, stem cell processing and infusion, neutropenic phase, engraftment, and post-engraftment period. The patient undergoes chemotherapy or radiation during the conditioning phase to create space or destroy the malignant cells. This could be achieved by the administration of cyclophosphamide, busulfan, and intravenous immunoglobulin (ALG) or by total lymphoid irradiation.

Low doses of chemotherapy and radiation are used, which does not eliminate all bone marrow cells. In this case the recipient benefits from the graft-versus-tumor effects of the nonmyeloablative transplant, and the recipients are treated with high doses of immunosuppressive agents during the early stages of the procedure. The result of this treatment is the presence of a state of mixed chimerism right after the procedure. The T cells from the donor marrow eradicate the stem cells of the recipient as the dose of immunosuppressive therapeutic regimen is decreased, which induces graft-versus-host disease and the graft-versus-tumor effect. There are a limited number of "niches" within marrow cavities and they are occupied, and this concern is addressed by using the conditioning phase to vacate sites. T cells are depleted to lessen the severity of the graft-versus-host disease. Neutropenic phase represents the period with an increased risk of infection due to a totally deficient immune response and a period of poor healing. This is followed by the engraftment phase where healing begins, and fever and infections are not a major problem with the exception of some viral infections. This period could last up to several weeks. This is the period where graft cells may mount a rejection response against the host (GVHD). Allogeneic bone marrow cells are readily rejected, and this could happen even if the host is minimally immunocompetent. The last phase is characterized as the post-engraftment period which could last from months to years. This phase manifests the development of tolerance, development of the immune system, and control of the chronic graft-versus-host disease. The rejection could result from acute or chronic graft-versus-host disease, which requires intensive immunosuppressive

therapy. Acute graft-versus-host disease is treated with high doses of corticosteroids, while chronic graft-versus-host disease can be treated by a variety of immunosuppressive therapeutic regimens. The various treatment options for graft-versus-host disease are shown in Fig. 8.3.

8.11.7 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation constitutes the transplantation of multipotent hematopoietic stem cells. They are isolated from bone marrow, peripheral blood, or umbilical cord blood. The transplantation could be autologous or allogeneic and is used for a variety of conditions, including malignant as well as nonmalignant diseases. For the malignant diseases, host's immune system is ablated with radiation or chemotherapy before the transplantation. A nonmyeloablative procedure has also been used that requires administration of low doses of chemotherapy and radiation. This procedure is indicated for elderly patients. Hematopoietic stem cell transplantation is indicated for many forms of leukemias, lymphomas, neuroblastoma, gliomas, myelodysplastic syndromes, thalassemia, aplastic anemia, and immune deficiency syndromes. It is also potentially beneficial in neurological disorders (Fig. 8.4). The procedure is most commonly used in patients with leukemia or multiple myeloma for whom chemotherapy is no longer a viable option. In pediatric cases, the patients are born with severe combined immunodeficiency or congenital neutropenia along with defective stem cells.

For autologous hematopoietic stem cell transplantation, hematopoietic stem cells are extracted from the patient and, after high-dose chemotherapy with or without radiation therapy, are administered back to the patient. As a result, the patients receive their own hematopoietic stem cells. In this case the recovery is rapid with lower chance of infection during immunocompromised state. The chances of rejection are also rare. As a consequence, it is a standard second-line treatment for lymphoma.

Allogeneic hematopoietic stem cell transplantation requires a foreign donor with matched tissue type to that of the recipient. HLA matching needs to be done for at least three or more loci. Acute graft-versus-host disease may develop within the first 3 months after allogeneic hematopoietic stem cell transplantation and will involve epithelial lining of skin, intestine, and liver. Chronic graft-versus-host disease may also develop, which is a major late-phase complication.

The outcome of hematopoietic stem cell transplantation is variable and depends upon many factors including type of the disease, stage of the disease, HLA matching, conditioning regimen, and source of stem cells. With availability of new immunosuppressive drugs and advances in technology, the survival rates have improved in patients receiving transplants.

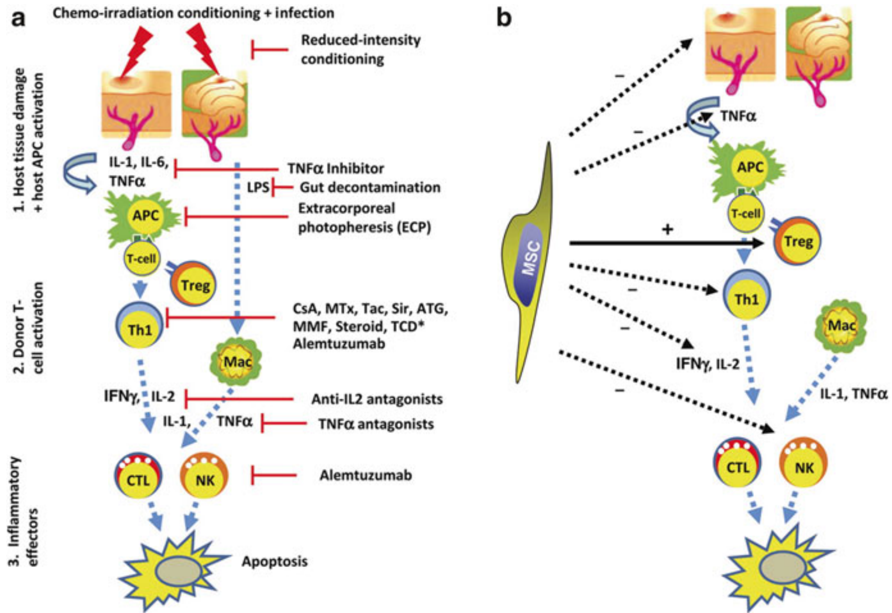

Fig. 8.3 The potential treatments of acute graft-versus-host disease: the subsection (**a**) depicts various drugs that are used to treat acute graft-versus-host disease. In part (**b**) of the figure, the proposed mechanisms of mesenchymal stem cells in treating acute graft-versus-host disease are shown (Reproduced with permission Source: Leung AYH, Kwong Y-L (2010) Hematopoietic stem cell transplantation: current concepts and novel therapeutic strategies. *British Medical Bulletin*. Issue: 1471–8391, doi:10.1093/BMB/LDP040, 93: 85–103, Oxford University Press)

8.12 Stem Cell Transplantation for Nonmalignant Diseases

Stem cells isolated from blood or bone marrow have exhibited therapeutic benefits in patients with certain autoimmune and cardiovascular disorders. They can be administered either as purified hematopoietic stem cells, mesenchymal stem cells, or as non-purified stems. Stem cells have the ability to self-renew and differentiate into desired cells. The presence of embryonic stem cells is transient during embryogenesis, although they can differentiate into all types of specialized cells. Adult stem cells are responsible to replace damaged or dying cells and are present all the time in adults. The potential for therapy is much greater for the embryonic stem cells, but they may also develop into tumor cells of any tissue type. The glycoproteins, $CD34^+$ or $CD133^+$ or both, are used to detect human hematopoietic progenitor cells. Most of these cells are in human blood or bone marrow and are committed progenitors. A very small number of $CD34^+$ or $CD133^+$ cells are stem cells, which repopulate throughout the life.

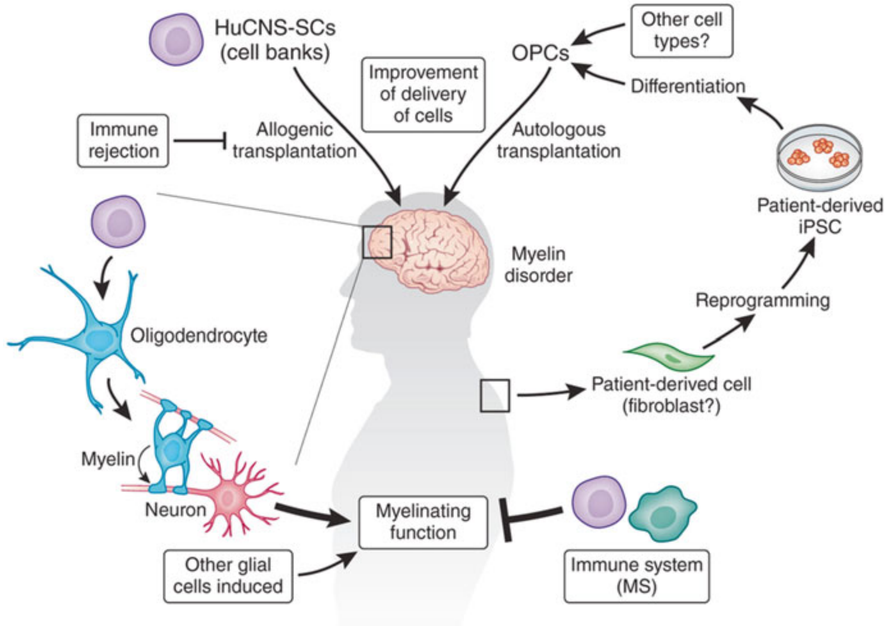

Fig. 8.4 Stem cell transplantation for neurological disorders: this figure depicts the potential applications of transplantation of human tissue-derived central nervous system stem cells, induced pluripotent stem cells, and oligodendrocyte precursor cells for the treatment of myelin disorders (Reproduced with permission. Source: Yang N, Wernig M (2013) Harnessing the Stem Cell Potential: a case for neural stem cell therapy. *Nature Medicine*. 19: 1580–1581. Nature Publishing Group)

Hematopoietic stem cell transplantation has been used in patients with severe autoimmune disorders. The logic behind it is to "terse" the immune system and create a new, non-autoimmune repertoire. But this procedure is associated with treatment-related mortality, which remains a concern and is not very widely used. Myeloablative or nonmyeloablative regimens have been used for autologous hematopoietic stem cell transplantation to treat autoimmune diseases. For autoimmune diseases nonmyeloablative regimens are used, whereas for cancer myeloablative procedures are employed. Nonmyeloablative procedure is used for autoimmune diseases to decrease mortality. The original standard nonmyeloablative drugs were comprised of cyclophosphamide and antithymocyte globulin. In recent years this combination has been replaced by cyclophosphamide and alemtuzumab. Another combination, which includes rituximab, cyclophosphamide, and antithymocyte globulin and called "rituximab sandwich," is also used. All these three combinations of treatments are called nonmyeloablative procedure.

Hematopoietic stem cell transplantation to treat autoimmune diseases is not considered a cure, but altering the progression of the disease. This transplantation has been used for systemic sclerosis, multiple sclerosis, rheumatoid arthritis, juvenile

idiopathic arthritis, Crohn's disease, type I diabetes, autoimmune-related retinopathy, optic neuropathy, autoimmune-related retinitis, optic neuritis, chronic inflammatory demyelinating polyneuropathy, celiac disease, neurovascular Sjögren syndrome, and dermatomyositis/polymyositis. Allogeneic human stem cell transplants by using sibling's hematopoietic stem cells have been also performed for treating autoimmune diseases. Allogeneic donors are intended for curing the disease as opposed to autologous transplants. The donor graft is made less immunogenic by lysing the T cells and therefore addressing the issue of the development of graft-versus-host disease in advance.

While the chances of removing the majority of T cells after autologous hematopoietic stem cell transplantation have been well confirmed, the relationship between regenerated T cells and the original baseline repertoire in terms of quantification is difficult to access. In a study, in patients with poor prognosis for multiple sclerosis undergoing autologous hematopoietic stem cell transplantation, distinct effects on $CD4^+$ and $CD8^+$ T cells repertoires were detected. All patients mostly developed a new repertoire for $CD4^+$ T cells, dominant T-cell receptor clones. The $CD4^+$ T-cell clones present before the procedure were not detected. However, this was not the case with dominant CD8+ T-cell clones, which were not ablated. The newly developed $CD8^+$ T-cell repertoire was the same as the clones present before the treatment procedure. There was reduced diversification of clones in patients who were not responsive to the transplant.

For a variety of neural diseases, the potential of stem cell-based transplantation is being considered. The targets for this therapy include Parkinson's disease, Batten's disease, acute or chronic brain damage, and substitution of oligodendrocytes (exogenous) for a plethora of neurological disorders. In comparison with complex neurons, oligodendrocytes may be preferable because their predominant function is to myelinate axons. Their precursor cells are present throughout the central nervous system and in large numbers. They possess the capacity to proliferate, migrate, and differentiate into myelinating oligodendrocytes in normal as well as disease-inflicted brain. This represents a therapeutic potential for utilizing exogenous neural stem cells or oligodendrocyte precursor cells when endogenous oligodendrocyte precursor cells are not able to function or myelinate. This would benefit a number of brain diseases, including multiple sclerosis where the capacity to myelinate is impaired. Other possibilities for the treatment may include cerebral palsy, spinal cord injury, and Pelizaeus–Merzbacher disease.

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Chapter 9
Acquired Immune Deficiency Syndrome

Abstract Acquired immune deficiency syndrome is caused by infection with HIV. The virus infects CD4$^+$ T cells causing their death and resulting in immunodeficiency, which leads to opportunistic infections that are the major cause of death. In this chapter, a brief history of the HIV infection is provided followed by the modes of HIV transmission, the criteria for diagnosis, a description of the components of the virus, and its cell cycle. The life cycle points to therapeutic targets to treat the infection. A major section of discussion is devoted to different classes of anti-HIV drugs which include HIV reverse transcriptase inhibitors, HIV protease inhibitors, HIV integrase strand transfer inhibitors, and inhibitors of viral binding. This description includes the mechanism of action of the drugs in each class, metabolism, drug–drug interaction, and side effects. The last section is devoted to the possibilities and obstacles for developing a vaccine designed to prevent and treat HIV infection.

Keywords Human immunodeficiency virus • HIV-1 • HIV-2 • SIV • *Pneumocystis carinii* pneumonia • Kaposi's sarcoma • gp120 • gp41 • gp160 • Gag • Pol • Env • Tat • Rev • Nef • Vif • Vpr • Vpu • Reverse transcriptase • Integrase • Ligase • Long-term nonprogressor • CD4 • α-Defensin protein • CD8$^+$ cells • Toxoplasmosis • Mycobacterial disease • Recurrent herpes simplex virus infection • Cytomegalovirus infection • K65R • L74V • M184V • Q151M • D67N • K219Q/e • L210W • M41L • T215Y/F • Reverse transcriptase inhibitors • Zidovudine • Didanosine • Zalcitabine • Emtricitabine • Lamivudine • Abacavir • Tenofovir • Efavirenz • Nevirapine • Delavirdine • Etravirine • Rilpivirine • HIV protease inhibitors • Saquinavir • Ritonavir • Indinavir • Nelfinavir • Lopinavir • Amprenavir • Fosamprenavir • Tipranavir • Atazanavir • Darunavir • HIV integrase inhibitors • Elvitegravir • Stribild • Dolutegravir • Enfuvirtide • Cobicistat • HIV vaccines • Gene editing

9.1 Introduction

In 1959 a Bantu man died of an unidentified illness in Belgian Congo, and later analysis of his blood samples confirmed him to be the first case of human immunodeficiency virus (HIV) infection. Human immunodeficiency virus 1 (HIV-1) and human immunodeficiency virus 2 (HIV-2), which cause AIDS, evolved from strains of simian immunodeficiency virus, SIVepz and SIVsm, respectively. SIVepz infects

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a subspecies of chimpanzee, and SIVsm infects sooty mangabeys, but AIDS is not observed in these hosts. Humans may have been exposed to SIV, but these strains are not able to adapt, infect, and transmit between humans. It is suggested that HIV-1 originated from a subspecies of chimpanzees as HIV-1 groups M and N have their origins in a distinct population of chimpanzees in Cameroon. The spread may have started after the Second World War. It is not known how many people developed this disease in the 1970s or before that, but in 1978 gay men in the United States and Sweden and heterosexuals in Haiti and Tanzania exhibited symptoms which were later identified as the acquired immune deficiency syndrome.

In 1981, cases of AIDS were diagnosed in young gay men in the United States, and the disease was called a gay-related immune deficiency (GRID). The physicians in New York and Los Angeles observed cases of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma which led the Centers for Disease Control and Prevention (CDC) to monitor young men, women, and babies with severe immunodeficiency. It was soon realized that this was not a local phenomenon but rather a global epidemic. Fourteen nations reported cases of AIDS in 1982, and at the same time, reports emerged of a hemophilic patient developing AIDS due to blood transfusion, as well as infected infants born to mothers with AIDS. It was not until late 1982 when this condition began to be diagnosed as AIDS, and in 1983, reports surfaced that the disease may be passed to women through heterosexual sex. In May 1983, Dr. Luc Montagnier of the Pasteur Institute in France reported that they have isolated a new virus, which may be responsible for AIDS that received little attention, and the virus was later named lymphadenopathy-associated virus or LAV. In 1984 Robert Gallo at the National Center Institute isolated human T-cell lymphotropic virus III (HTLV-III), and this virus was considered to be responsible for AIDS. However, in 1985, LAV and HTLV-III were identified to be the same, and in 1986, it was renamed human immunodeficiency virus or HIV. Attempts were underway to develop a test which could detect the presence of the virus in the blood, since its transmission was not accompanied by immediate symptoms for diagnosis.

AIDS is now pandemic, and according to the Joint United Nations Program on HIV/AIDS, 74 million people have contracted HIV virus and 39 million have died worldwide. In the twenty-first century, it remains an ultimately fatal disease that is difficult to treat, but the development of new drugs has improved the outlook of patients infected with the virus in the United States and Western Europe.

9.2 Human Immunodeficiency Virus (HIV)

AIDS is a result of immunodeficiency attributed to the HIV that is a lentivirus, which in contrast to the herpes viruses replicates constantly. There is not a period of viral latency after infection, unless some infected cells contain nonreplicating virus. HIV only infects humans and chimpanzees and has two major families, HIV-1 and HIV-2. HIV-1 is responsible for the spread of the infection worldwide, while HIV-2 is endemic in Western Africa. HIV-1 and HIV-2 share about 40 % of the genome. HIV-1

has at least five subfamilies or clades. An HIV particle is about 100–150 billionths of a meter in diameter, it is a retrovirus surrounded by a viral envelope (coat of lipid bilayer) that projects spikes, gp120 and gp41 proteins. A layer called the matrix, which is made up of the protein p17 is present under the viral envelope. The viral core (capsid) is composed of protein p24 in which are contained three enzymes, reverse transcriptase, integrase, and protease, which are required for HIV replication. Two copies of genome (RNA) are contained in the nucleocapsid core. HIV is composed of nine genes, three of which are the structural genes called gag, pol, and env. Gag encodes major structural proteins of the virus, pol encodes the reverse transcriptase, the proteases, and the viral integrase; and env encodes the proteins that are responsible for the attachment of the virus and entry to the cell. The other six genes, tat, rev, nef, vif, vpr, and vpu, are responsible for the translation of regulatory proteins required for infection. The regulatory proteins enhance virion production and counter host defense. A sequence called the long-term repeat is present on each of the RNA strands and serves as a control mechanism for HIV replication (Fig. 9.1).

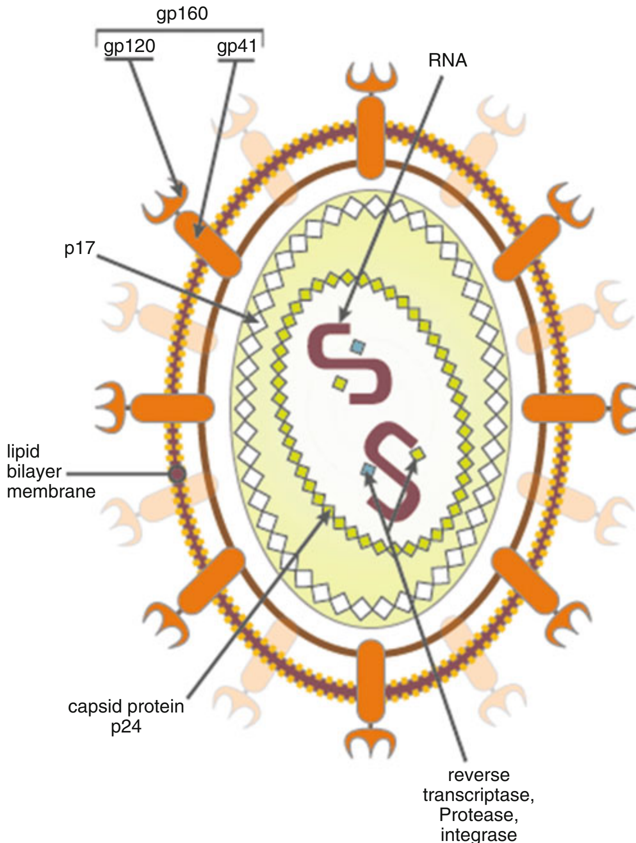

Fig. 9.1 Human immunodeficiency virus, a schematic representation

9.2.1 Human Immunodeficiency Virus Replication

The replication of HIV takes place only in the human cell. The process begins after the recognition of CD4 molecule on human cells by the virus, and the glycoprotein gp120, along with gp41, infects any target cell that expresses CD4 receptors. The gp120 portion binds to CD4 with high affinity, whereas gp41 mediates fusion of the virus with the membrane of the target cell. The interactions involve the trimeric envelope complex (gp160), CD4, and a chemokine receptor. The chemokine receptors are generally either CCR5 or CXCR4, but others may also interact. The gp120 and gp41 portions collectively form gp160 spike with three transmembrane glycoproteins (gp41) and three extracellular glycoproteins (gp120). The fusion initiates with the high-affinity binding of CD4-specific domains of gp120 to the N-terminal membrane distal domain of CD4. After the binding of gp120 to CD4, there is a structure change in the envelope resulting in the exposure of the chemokine-binding domain of gp120. This allows the gp120–CD4 complex to interact with chemokine receptors resulting in the penetration of gp41 into the cell membrane. A hairpin structure is formed as a result of collapse of the extracellular portion of gp41, which assists the virus in entering the host cell (Fig. 9.2).

After the entry of the virus into the cell, single-stranded RNA is separated from the viral proteins, and cDNA is then transcribed from RNA, both by reverse transcriptase. A second strand of cDNA is synthesized to make double-stranded viral DNA, and this viral double-stranded DNA may remain in the cytoplasm as unintegrated circular DNA or migrate to the nucleus. In the nucleus, a complete genomic copy of viral DNA is integrated into the host cell genome by viral integrase. After integration, infection can be latent without active viral replication, which requires upregulation of T cells. The upregulation of T cells results in the activation of transcription factor NF-κB (nuclear factor-kappa B), which is required to actively produce the HIV. The mRNA is made from the integrated provirus and is spliced into smaller chunks, which produce Tat and Rev that are two regulatory proteins. Tat stimulates new virus production, and Rev inhibits mRNA splicing. Full-length mRNA now produces structural proteins including Gag and Env, which surround the genomic RNA, and nucleocapsids are formed. At the cell surface, the transmembrane envelope and structural proteins assemble, which are concentrated in cholesterol-rich lipid rafts that results in the packaging of new virus particles. The final step in this process is assembly and release, as the plasma membrane of the host cell is the site of the assembly of the new HIV virions. After migrating through the endoplasmic reticulum, the Env polyprotein (gp160) enters the Golgi complex where protease processes gp160 into two glycoproteins, gp41 and gp120. These molecules are transported into the cell membrane of the virus and Gag (P55) as well as Gag–Pol (P160) proteins associate on the inside of the membrane. The polyproteins of HIV are cleaved into individual functional HIV proteins and enzymes by HIV proteases during maturation. The viral particles are now completely assembled and are released by budding from the host cells.

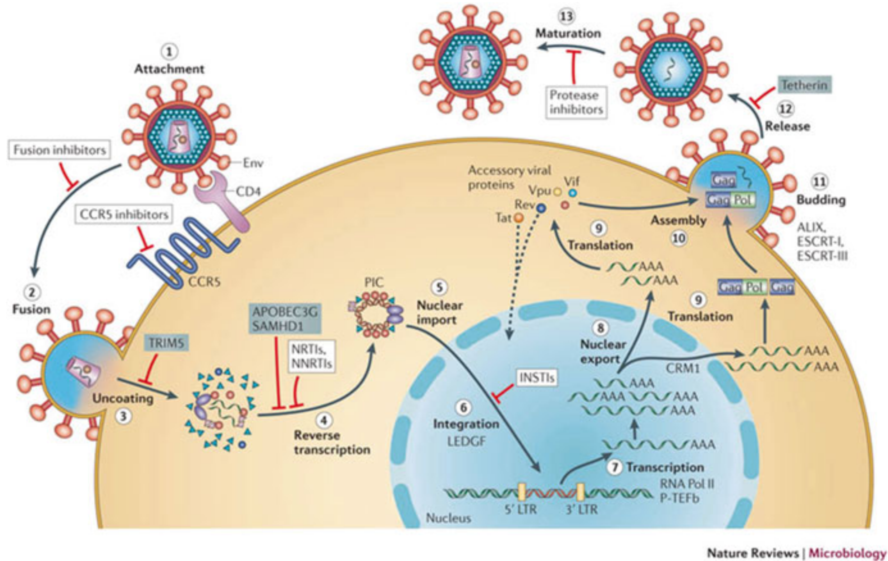

Fig. 9.2 Life cycle of HIV. HIV infects the CD4$^+$ T cells by binding of gp120 to CD4. The gp41 is responsible for the fusion of the two membranes. The interaction involves three molecules gp160 (combination of gp120 and gp41), CD4, and a chemokine (CCR5 or CXCR4) receptor. After fusion viral genome enters the cell. Reverse transcriptase then copies viral RNA genome into double-stranded cDNA. This is followed by the migration of viral cDNA into the nucleus where it is integrated into host DNA with the help of viral ligase and remains dormant until the T cell proliferates. Pre-integration-associated integrase is responsible for the formation of integrated provirus. Host chromatin-binding protein lens epithelium-derived growth factor also aids in this process. T-cell activation causes low-level transcription of provirus. Host polymerase RNA II and positive transcription elongation factor b are involved in the production of viral mRNAs of various sizes. The larger viral mRNA is dependent on energy and host's essential nuclear protein CRM1. This is followed by multiple splicing of RNA transcripts which allows translation of early genes tat and rev. Tat is responsible for amplifying transcription of viral RNA; viral RNA causes the transport of single-spliced or unspliced viral RNA to cytoplasm. The next step is the translation of late proteins Gag, Pol, and Env and assembly into viral particles. The last step is the budding and protease-mediated maturation which forms the infectious viral particles. Budding requires endosomal sorting complex required for transport and ALIX (Reproduced with permission. Source: Engelman A, Cherepanov P (2012) The structural biology of HIV: mechanistic insights. *Nature Review Microbiology* 10: 279–290. Nature Publishing Group)

9.2.2 *Human Immunodeficiency Virus and Disease*

HIV spreads by contact with body fluids, and the common modes of infection include sexual intercourse and contaminated needles used for intravenous drug delivery. Previously, therapeutic uses of infected blood and infected breast milk for the baby were also modes of transmission but both have declined, due to better screening for blood as well as AIDS education for the expectant mothers. The virus is carried in infected CD4$^+$ T cells and macrophages or as free viruses in the blood, semen, vaginal fluids, and milk.

In general, the viral infections are acute and limited, resulting in the development of lasting protective immunity. This is not the case with HIV, and an immune response is rarely developed that could completely eliminate the virus. Nonetheless, during the first few months after the HIV infection, most people mount an immune response, which includes both the humoral and cellular responses. During this phase, the number of $CD8^+$ cells increases up to 20-fold above the normal levels, but the levels of $CD4^+$ cells fall sharply in the first few weeks after infection resulting in the suppression of immune response induced by $CD4^+$ cells. The individuals who can produce HIV-specific $CD4^+$ cells for a longer duration have lower viral load than the individuals who cannot produce a healthy HIV-specific $CD4^+$ cell response. Due to their ability to produce interferon-γ and IL-2, the individuals producing HIV-specific $CD4^+$ cell response can effectively control HIV loads. HIV can alter the function of $CD4^+$ cells even without infecting them.

During the early phase of infection, HIV-specific $CD8^+$ cells are produced and control the viral load, but within weeks they die and only memory $CD8^+$ cells are left. Only in some individuals a strong HIV-specific $CD8^+$ cell's response continues to exist resulting in a good control of the viral load. The gradual loss of HIV-specific CD8+ function may result from a continued mutation and high levels of viral turnover. This gradually diminishes the capability of the cell to recognize the genetic sequences of the virus, due to killing of some of the CD8 T cells' repertoire by HIV itself.

Four to eight weeks after HIV infection, antibody responses begin to develop, they are predominantly directed against the circulating virus, and some antibodies may destroy the virus-infected cells as well. However, the antibody response is unable to continue to neutralize HIV because of the rapid mutation of the virus. The development of the initial cellular and humoral response leads to a clearance of much of the viremia and a rebound of $CD4^+$ cells.

After a period of apparent quiescence of the disease, known as clinical latency or the asymptomatic period, most of the patients who are infected with HIV will eventually develop AIDS. But this is not a silent period because there is a gradual decline in the number and function of $CD4^+$ cells. If the number of peripheral $CD4^+$ cells falls less than 200 cells/mm^3, the risk of opportunistic infection and ultimate death increases. The opportunistic infections appear anywhere between 3 and 15 years or more after the primary infection, but some patients who are characterized as long-term nonprogressor (LTNP) have demonstrated the ability to be infected with the virus for more than 20 years without significant decline in CD4 count or function or any other symptoms. These patients are not a homogenous group based on the viral load and specific immune response against HIV as some in this group are infected with an inefficiently replicating virus while others are infected with normal replicating HIV. The patients in LTNP group who are infected with normal replicating virus produce robust humoral and cell-mediated immunity. A subgroup of LTNP exhibit signs of progression without any changes in clinical and laboratory parameters over an extended period and are called long-term survivors (LTSs). The α-defensin protein may play a role in the inactivation of HIV virus, and CD38 subset of CD8 T cells may play a role in the progression of the disease as nonprogressors have lower levels of CD38 subset of $CD8^+$ cells. The regulatory T cells accumulate in the

lymphoid tissues of patients infected with HIV. The interaction between regulatory T cells and HIV promotes the survival of regulatory T cells resulting in the inhibition of cell-mediated immune response. The CD25$^+$ regulatory T cells obtained from the peripheral blood and the lymph nodes of the patients infected with HIV who have high viral load and/or low CD4$^+$ T-cell count are potent suppressors of HIV-specific CD8$^+$ T cells.

9.2.3 Human Immunodeficiency Virus Transmission

Most commonly HIV is spread among adults through sexual intercourse. The entry points of the virus include the rectum, penis, vagina, vulva, and mouth (after oral sex). The damage to the lining of these organs may increase the chances of acquiring the infection. Other sexually transmitted infections that may result in ulcers or inflammation also increase the likelihood of transmission. The dendritic cells may initiate the transmission process, which carry the virus from the point of its origin to the lymph nodes where HIV infects other lymphoid cells. DC-SIGN is a type II transmembrane protein with an external C-type lectin domain expressed on the surface of several subsets of immature and some mature dendritic cells. This DC-SIGN is implicated in this process.

After reaching the lymph nodes, CD4$^+$ cells are rapidly infected, and replication of the virus continues. During the initial phase of infection, the virus is spread throughout the body via the blood which contains many viral particles. The flu-like symptoms are first observed in about 70 % of the patients 2–4 weeks after HIV infection. At this stage HIV titer is reduced due to the development of virus-specific CD8$^+$ cells and due to humoral immune response that generally causes a return to the normal numbers of CD4$^+$ cells. As the HIV continues to replicate, a person may stay free of HIV-related symptoms for years. The high rate of mutation makes it impossible for the body to completely get rid of the HIV. Independent of mutation, certain subsets of HIV recognizing killer T cells are not present or lack optimal function. There is an inhibition of interferon secretion and cytotoxic T-cell activity due to impairment in the function of CD4$^+$ cells. The HIV is also protected by the immune surveillance when it hides within the chromosomes of the infected cells.

Several billion virus particles are produced every day as a result of rapid replication of HIV, and a wide range of variants are also produced due to the recombination among various strains of HIV. The strains of virus in the same person during early and late stages of infection differ in their virulence. They infect and kill different cells. As the disease progresses, the virulence increases and the spectrum of the target cells widens, which may be due to the ability of virus in utilizing additional co-receptors for infection as it gets to the later stages.

Different mechanisms have been proposed regarding the ability of HIV to destroy or disable CD4$^+$ cells. A very large number (billions) of CD4$^+$ cells are destroyed by HIV every day, and finally the body is unable to regenerate these cells. The proposed mechanisms include direct T-cell killing, apoptosis, innocent bystanders, anergy,

and damage to precursor cells. Direct T-cell killing is a result of HIV which infects $CD4^+$ cells, replicates in them, and interferes with their normal function. The presence of HIV proteins in $CD4^+$ cells and on the surface of uninfected cells lead to apoptosis. Uninfected $CD4^+$ cells are also killed by antibody-mediated cell-dependent cytotoxicity, since they exhibit HIV particles on their cell surface. Cytotoxic T cells ($CD8^{+)}$ are also involved in the killing of HIV-infected $CD4^+$ cells. It is interesting to note that while HIV also infects monocytes and macrophages, it does not kill these cells, and infected monocytes and macrophages carry HIV infection to the brain.

Although the net effect of HIV infection is immunodeficiency, the infection generally results in the increased activation of the immune response, but the overall impact of this immune hyperactivity is negative. The activated $CD4^+$ cells play a pivotal role in the replication of the virus; this activation of $CD4^+$ cells enhances the secretion of a variety of cytokines, some of which contribute to muscle wasting. A super active humoral response against HIV impairs the body's ability to mount antibody response against other pathogens, and the activation of immune response by other pathogens further induces HIV replication in the infected individuals. All of these events induce the progression of the HIV disease.

9.2.4 Criteria for Diagnosis

According to the Centers for Disease Control and Prevention (CDC), the diagnosis of AIDS constitutes certain opportunistic infections, neoplasms, encephalopathy, or wasting syndrome in the presence of HIV infection. In 1993, the CDC expanded the criteria to also include $CD4^+$ T-cell count below 200 cells per microliter in the presence of HIV infection. The most common opportunistic infections include *Pneumocystis carinii* pneumonia, pneumonitis, toxoplasmosis, mycobacterial disease, recurrent herpes simplex virus infection, and/or cytomegalovirus infection. Kaposi's sarcoma is the most common form of cancer. HIV-related nervous system diseases include acute septic meningitis, AIDS dementia complex, subacute encephalitis, HIV encephalopathy, and CNS opportunistic infections and neoplasm.

9.3 Clinical Strategies for the Treatment of Aids

The treatment for AIDS is referred to as highly active anti-retroviral therapy (HAART). This consists of a combination of drugs that include generally two nucleoside reverse transcriptase inhibitors in combination with an integrase strand transfer inhibitor, a non-nucleoside reverse transcriptase inhibitor, or a protease inhibitor, plus a pharmacokinetic enhancer (cobicistat or ritonavir). The drug selection is important based on increasing the potency, reducing the side effects, monitoring or preventing cross-resistance, and focusing on the future viable therapeutic options as a result of the drug resistance.

Resistance to anti-retroviral therapy cannot be avoided. The HIV reverse transcriptase is error-prone and lacks a proofreading function. A high number of replication cycles and the presence of low drug concentrations further contribute to the mutations. These mutations alter the enzymes such as reverse transcriptase, and as a consequence, the respective drugs are unable to function optimally. The number of mutations which results in resistance is different for each drug.

The binding of nucleoside reverse transcriptase inhibitor into viral DNA causes the termination of DNA chain and inhibits additional elongation of DNA. The non-nucleoside reverse transcriptase inhibitors bind to the hydrophobic pocket within the p66 subunit of the enzyme and inhibit reverse transcription. There are two different mechanisms that contribute to the resistance to nucleoside reverse transcriptase inhibitors. One mechanism is discrimination, in which case the viral reverse transcriptase will not bind to nucleoside reverse transcriptase inhibitor. Mutations such as K65R, L74V, M184V, and Q151M utilize this mechanism. For the second mechanism the mutated reverse transcriptase causes the phosphorolytic excision of nucleoside reverse transcriptase inhibitor from the 3′ primer end of the viral DNA. This process is referred to as primmer unblocking. Some examples of thymidine analog mutations include D67N, K219Q/e, L210W, M41L, and T215Y/F.

The first-generation non-nucleoside reverse transcriptase inhibitors have a low genetic barrier to resistance. Only a single amino acid substitution in the reverse transcriptase enzyme can produce substantial resistance. The degree of cross-resistance is also high in this group. The binding pocket of the non-nucleoside reverse transcriptase inhibitors is predominantly present in p66 subunit of reverse transcriptase. There are three mechanisms involved in the non-nucleoside reverse transcriptase inhibitor mutations. They include blocking the entry of the inhibitor into the respective binding pocket, affecting interaction between the drug and the residues that line the respective binding pocket, and/or altering the conformation of the drug-binding pocket. However, additional mechanisms could also be involved.

9.4 Reverse Transcriptase Inhibitors

9.4.1 Nucleoside Reverse Transcriptase Inhibitors

9.4.1.1 Zidovudine (AZT)

Zidovudine (3′-azido-3′-deoxythymidine) is a thymidine analog that inhibits the activity of the reverse transcriptase in HIV-1, HIV-2, and in a number of other retroviruses. It needs to be phosphorylated by cellular thymidine kinase, after it diffuses into the cell, before it could exert its inhibitory effect on the reverse transcriptase. Its selectivity is attributed to its greater affinity of HIV reverse transcriptase than for human DNA polymerase. Thymidine kinase phosphorylates zidovudine to zidovudine 5′-monophosphate, which is phosphorylated by thymidylate kinase to zidovudine 5′-diphosphate followed by its phosphorylation by nucleoside diphosphate

kinase to the pharmacologically active zidovudine 5′-triphosphate. Thymidine kinase is S-phase-specific, which causes zidovudine to be more potent in activated lymphocytes as opposed to the resting cells. It has higher activity against lymphoblasts and monocytes than the cells previously infected. Zidovudine's principle target is the lymphocytes in which they are more active than the macrophages. It has no effect on cells that are already infected with the virus. Zidovudine also becomes incorporated into the transcribed DNA strand and, as a consequence, further prevents HIV–DNA synthesis. The resistance to the drug results from site-directed mutagenesis at codons 41, 44, 67, 70, 118, 210, 215, and 219 of viral reverse transcriptase.

Zidovudine is rapidly absorbed with an oral bioavailability of 60–70 %. The peak plasma concentrations are reached within 1 h. The absorption varies widely, and food intake has a retarding effect. The elimination half-life of the triphosphate metabolite is 3–4 h as opposed to the parent compound, which is only 1 h. It is metabolized in the liver and is converted to glucuronide form, 5-glucuronyl zidovudine. Zidovudine is used for the treatment of HIV infection or exposure in adults, children, and pregnant mothers (to prevent mother-to-child transmission). For the effective treatment, the drug is always administered in combination with other anti-HIV drugs. As a monotherapeutic agent, it is 67 % effective to control the risk of transmission from infected pregnant mother to fetus.

Side Effects and Drug Interactions

During initiation of the zidovudine therapy, the common side effects include severe headache, nausea, emesis, fatigue, malaise, and myalgia, but these symptoms diminish with time. Other side effects include nail pigmentation, esophageal ulceration, hepatitis, neurotoxicity, and bone marrow suppression.

Fluconazole, probenecid, and atovaquone increase the risk of myelotoxicity by zidovudine. This may be attributed to an increased plasma concentration of zidovudine in the presence of these drugs, perhaps through their inhibitory effects on glucuronosyl transferase. Rifabutin and rifampin decrease plasma concentrations, and clarithromycin decreases the absorption of zidovudine.

9.4.1.2 Didanosine (Dideoxyinosine, DDI)

Didanosine is a synthetic purine nucleoside analog that inhibits the activity of reverse transcriptase in HIV-1, HIV-2, other retroviruses, and zidovudine-resistant strains. A nucleobase carrier helps transport it into the cell where it needs to be phosphorylated by 5′-nucleotidase and inosine 5′-monophosphate phosphotransferase to didanosine 5′-monophosphate. Adenylosuccinate synthetase and adenylosuccinate lyase then convert didanosine 5′-monophosphate to dideoxyadenosine 5′-monophosphate, followed by its conversion to diphosphate by adenylate kinase and phosphoribosyl pyrophosphate synthetase. This is then phosphorylated by

creatine kinase and phosphoribosyl pyrophosphate synthetase to dideoxyadenosine 5′-triphosphate that is the active reverse transcriptase inhibitor. Dideoxyadenosine triphosphate inhibits the activity of HIV reverse transcriptase by competing with the natural substrate, deoxyadenosine triphosphate, and its incorporation into viral DNA causes termination of viral DNA chain elongation. It is 10- to 100-fold less potent than zidovudine for its antiviral activity, but is more active in non-dividing and quiescent cells than zidovudine. At clinically relevant doses, it is not toxic to hematopoietic precursor cells or lymphocytes, and the resistance to the drug results from site-directed mutagenesis at codons 65 and 74 of viral reverse transcriptase.

The oral bioavailability of didanosine is about 35–45 %. Food may decrease its absorption by 50 % or more. As a consequence, it should be administered a minimum of 30 min before or 2 h after eating. Peak plasma concentrations are reached within 1 or 2 h of administration after a chewable tablet and delayed release capsule, respectively. The elimination half-life of the triphosphate metabolite is 35–40 h as opposed to the parent compound, which is about 1½ h. The drug is not metabolized significantly and is excreted by glomerular filtration and tubular secretion. Didanosine is used in combination with other anti-retroviral agents for HIV-infected adults and children.

Side Effects and Drug Interactions

The major dose-limiting toxicities of didanosine include peripheral neuropathy and pancreatitis. The neuropathy is typically a symmetrical distal sensory neuropathy that is reversible and typically causes paresthesias, numbness, and pain in lower extremities. Didanosine also causes retinal changes and optic neuritis. Other adverse effects include diarrhea, skin rash, headache, insomnia, seizures, hepatic toxicity, elevated hepatic transaminases, and asymptomatic hyperuricemia.

Allopurinol increases didanosine plasma concentrations, and their coadministration is not recommended. Ganciclovir, tenofovir, and disoproxil also increase didanosine plasma concentrations, and dose reduction is recommended. Conversely, methadone decreases didanosine plasma concentrations, and appropriate doses for the combination have not been established. Didanosine should not be administered with drugs which cause pancreatic or neurotoxicity. Ribavirin increases its risk of toxicity and should not be coadministered.

9.4.1.3 Zalcitabine

Zalcitabine (2′, 3′-dideoxycytidine), a reverse transcriptase inhibitor, is a synthetic pyrimidine nucleoside analog that is active against HIV. Its entry into the cell is by carrier-mediated transport and passive diffusion. Inside the cell deoxycytidine kinase converts it to dideoxycytidine 5′-monophosphate, which is then converted to diphosphate by deoxycytidine monophosphate kinase, followed by its conversion by nucleoside diphosphate kinase to dideoxycytidine 5′-triphosphate, which

is the active metabolite. Zalcitabine is more potent in resting cells than other nucleoside analogs, because it is more efficiently phosphorylated in non-dividing cells. It inhibits reverse transcriptase by competing with deoxycytidine 5′-triphosphate, and it also incorporates into viral DNA resulting in the termination of viral DNA growth. Dideoxycytidine 5′-triphosphate also inhibits the activity of cellular DNA polymerase-β and mitochondrial DNA. The resistance to the drug results from site-directed mutagenesis at codons 65, 69, 74, and 184, of viral reverse transcriptase.

After oral administration, the bioavailability of zalcitabine is more than 80 %. Food slightly interferes with its absorption. Sixty to 80 % of the compound is excreted unchanged in the urine. Its (dideoxycytidine 5′-triphosphate) peak concentrations are at 2–3 h. The primary metabolite is dideoxyuridine, which is <15 % of the administered dose. Zalcitabine is indicated in combination with other anti-retroviral agents for the treatment of HIV infection.

Side Effects and Drug Interactions

The adverse effects of zalcitabine include peripheral neuropathy, oral ulceration, and stomatitis. Additional side effects may include elevated hepatic transaminases, arthralgias, myalgias, fatigue, headache, fever, and cardiomyopathy. It does not interact with zidovudine, and lamivudine inhibits its phosphorylation. Zalcitabine should not be administered with other drugs that cause neuropathy or pancreatitis, including didanosine and stavudine.

9.4.1.4 Emtricitabine

Emtricitabine, a reverse transcriptase inhibitor, is a synthetic nucleoside analog with activity against HIV-1, HIV-2, and HBV. Following its entrance into the cell by passive diffusion, deoxycytidine kinase and cellular kinases phosphorylate it into emtricitabine 5′-triphosphate. It is the active form of the drug that competes with the natural substrate of the reverse transcriptase, incorporates into viral DNA, and terminates the elongation of viral DNA. Emtricitabine has low affinity for human DNA polymerases. Since it is chemically related to lamivudine, both drugs share a number of properties including the development of site-directed mutagenesis of viral reverse transcriptase at codon 84, where the single amino acid substitution changes the methionine residue to valine.

After oral administration, emtricitabine is rapidly absorbed with a bioavailability of 93 %, and it could be administered with or without food. The peak plasma concentration occurs 1–2 h after the oral dose. It does not significantly bind to plasma proteins, and its elimination half-life is 8–10 h. Following glomerular filtration and active tubular secretion, it is primarily excreted unmetabolized in urine. In combination with other anti-retroviral agents, emtricitabine is recommended for the treatment of HIV infection.

Side Effects and Drug Interactions

The toxicity of emtricitabine is rather minimal. The side effects include hepatitis, pancreatitis, hyperpigmentation of the skin, elevated hepatic transaminases, headache, diarrhea, and nausea. Skin discoloration has been reported in high frequency in emtricitabine-treated groups, rather than the control group. There are no reports regarding adverse drug interactions with emtricitabine.

9.4.1.5 Lamivudine

Lamivudine (-2′, 3′-dideoxy, 3′-thiacytodine), a reverse transcriptase inhibitor, is a synthetic nucleoside analog with activity against HIV-1, HIV-2, and HBV. It enters the cell via passive diffusion, where deoxycytidine kinase converts it to its monophosphate form that is then converted to the diphosphate by deoxycytidine monophosphate kinase. This is followed by its conversion by nucleoside diphosphate kinase to lamivudine 5′-triphosphtate, which is the active form of the drug. It inhibits reverse transcription via DNA chain termination of the nucleotide analog into viral DNA. The resistance to the drug results from site-directed mutagenesis of viral reverse transcriptase with single amino acid substitutions, M184V and M184I, changing the methionine residue to either valine or isoleucine.

After oral administration, lamivudine is rapidly absorbed, and its bioavailability is about 86 %. Food slows down its absorption, and it is excreted unchanged in the urine. The drug crosses the placenta. Its concentrations are higher in the male genital tract in comparison with the circulation. In combination with other anti-retroviral therapy, lamivudine is recommended for the treatment of HIV infection, in patients who are negative for HLA-B5701. It inhibits plasma HIV-1 RNA concentrations, but resistance develops rapidly when used as monotherapy.

Side Effects and Drug Interactions

The toxicity of lamivudine is rather minimal. The side effects include headache, nausea, neutropenia, malaise and fatigue, nasal symptoms, and cough. Pancreatitis has been reported in children, which has been fatal in some cases. Lamivudine and emtricitabine are not used in combination since they are similar in their pharmacologic effects and in the development of the resistance. Since lamivudine is predominantly excreted in urine, its concurrent administration with drugs that utilize the same pathway for clearances should be carefully monitored. Lamivudine is also an inhibitor of the phosphorylation of zalcitabine.

9.4.1.6 Abacavir

Abacavir [(15, CIS)-4-[amino-6-(cyclopropylamino)-9H-purin-9-yl] 2-cyclopentene-1-methanol], a reverse transcriptase inhibitor, is a synthetic carbocyclic nucleoside analog with activity against HIV. After it enters the cells,

adenosine phosphotransferase converts it to a monophosphate that is then converted to (−) carbovir3′-monophosphate. It is then converted to di- and triphosphate by cellular kinases. The triphosphate is the active form of the drug, which inhibits the activity of HIV reverse transcriptase by competing with the natural substrate GTP. It also incorporates into viral DNA and terminates the elongation of viral DNA. Abacavir is a weak inhibitor of human DNA polymerase α, β, and γ. The resistance to the drug results from site-directed mutagenesis of viral reverse transcriptase. The specific codon substitutions include K65R, L74V, Y115F, and M184V. Furthermore, mutations at codons 41, 210, and 215 have also been reported.

After oral administration, abacavir is rapidly absorbed, and its bioavailability is about 83 %. Food does not interfere with its absorption, and it is metabolized by alcohol dehydrogenase to 5′-carboxylic acid derivative and to 5′-glucuronide by glucuronidation. Abacavir does not affect the cytochrome P450 system. In combination with other anti-retroviral drugs, abacavir is indicated for the treatment of HIV-1 infection. It is a potent nucleoside reverse transcriptase inhibitor that reduces HIV plasma concentration and increasing $CD4^+$ count.

Side Effects and Drug Interactions

The most serious and sometimes fatal side effect associated with abacavir is hypersensitivity reaction, which is a multi-organ clinical syndrome involving one or more of the following symptoms: fever, rash, gastrointestinal problems (nausea, vomiting, diarrhea, and abdominal pain), constitutional component (malaise, fatigue, aches, and pain), and/or respiratory component (cough, dyspnea, pharyngitis). The onset of symptoms has a median time of about 11 days; in most cases, the hypersensitivity reaction takes place within 6 weeks. If the symptoms appear, the drugs need to be immediately discontinued and can never be resumed. The hypersensitivity reaction to abacavir is associated with HLA-B5701 gene, and the reaction takes place in 2–9 % of the patients, which results in death in 4 % of the patients. The other serious side effects associated with abacavir alone or in combination with other nucleoside analogs are lactic acidosis and severe hepatomegaly with steatosis; some of these cases may be severe and fatal. There are no serious drug interactions associated with abacavir, with the exception that high ethanol consumption may increase its plasma levels and effects its elimination.

9.4.2 Non-nucleoside Reverse Transcriptase Inhibitors

9.4.2.1 Tenofovir

Tenofovir disoproxil fumarate is a prodrug that is a fumaric acid salt of bis-isopropoxycarbonyloxymethyl ester derivative of the active compound and requires initial hydrolysis and is converted to tenofovir. It is a reverse transcriptase inhibitor,

which is a nucleotide analog, active against HIV-1, HIV-2, and HBV. Cellular kinases then phosphorylate it to tenofovir diphosphate, which inhibits the activity of HIV reserve transcriptase by competing with the natural substrate of the reverse transcriptase deoxyadenosine 5′-triphosphate. It also incorporates into viral DNA and terminates the elongation of viral DNA. The affinity of tenofovir is low for human DNA polymerases α, β, and γ. The resistance to the drug results from site-directed mutagenesis of viral reverse transcriptase; only one codon, K65R, is involved in resistance.

The oral availability in the absence of food is 25 % that is increased after a meal, which is high in fat content (700–1000 cal containing 40–50 % fat). Its plasma half-life is 14–16 h. Tenofovir is not a substrate of cytochrome P450 system. After an IV administration, 70–80 % is found unchanged in urine. Its elimination is by glomerular filtration and active tubular secretion. Therefore, its concurrent administration with drugs that utilize the same pathway for clearance should be monitored. In combination with other anti-retroviral agents, tenofovir is indicated for the treatment of HIV infection.

Side Effects and Drug Interactions

Adverse effects are not a major concern with the use of tenofovir. Only rare incidence of acute renal failure and Fanconi syndrome may result. A risk of lactic acidosis and severe hepatomegaly with steatosis, including fatal cases, have also been observed. A combination of tenofovir and didanosine is not recommended since tenofovir increases the AUC of didanosine. It also reduces the AUC of atazanavir.

9.4.2.2 Efavirenz

Efavirenz is a non-nucleoside reverse transcriptase inhibitor specific for HIV-1. After binding to a site distant from the active site on the HIV-1 reverse transcriptase, it disrupts catalytic activity of the enzyme by causing a conformational change and does not compete with deoxynucleoside triphosphates. Efavirenz does not inhibit HIV-2 reverse transcriptase and human DNA polymerases α, β, γ, and δ. The resistance to the drug develops rapidly from site-directed mutagenesis specifically at codon 103 but also at codons 100, 106, 108, 181, 190, and 225 of viral reverse transcriptase. This resistance will be applicable for all non-nucleoside transcriptase inhibitors.

After oral administration, efavirenz is rapidly absorbed from the GI tract, and the peak plasma levels are attained within 5 h, but at higher doses (>1600 mg), the absorption is diminished, and high-fat meals increase the bioavailability up to 22 %. It is highly bound (99.5 %) to plasma proteins predominantly albumin and is metabolized by the cytochrome P450 system (CYP3A4, CYP2B6). Efavirenz is hydroxylated, and hydroxylated metabolites undergo glucuronidation. It induces its own metabolism by the activation of the cytochrome P450 system. The half-life after a

single dose is 52–76 h and after multiple doses is 40–55 h. It is excreted both in the urine and the feces. In combination with other anti-retroviral agents, efavirenz is recommended for the treatment of HIV infection.

Side Effects and Drug Interactions

The most serious side effects of efavirenz involve psychiatric symptoms, rash, and nervous systems. The psychiatric symptoms include suicide thoughts, depression, paranoia, manic disorders, and aggressive behavior. The rashes include maculopapular skin eruptions. Life-threatening Stevens–Johnson syndrome has also been reported. The neurological symptoms are difficulty in concentration, insomnia, dizziness, confusion, agitation, hallucinations, and amnesia. Additional side effect may include an increase in cholesterol and hepatic transaminase levels.

Efavirenz inhibits the plasma levels of indinavir, saquinavir, and amprenavir and increases the concentrations of ritonavir and nelfinavir. It also lowers the plasma levels of methadone, phenytoin, carbamazepine, and phenobarbital. Drugs that stimulate cytochrome P450 system will increase its clearance and should not be coadministered.

9.4.2.3 Nevirapine

Nevirapine is a member of the dipyridodiazepinone class of chemicals and is a non-nucleoside reverse transcriptase inhibitor, which induces a conformational change in HIV-1 reverse transcriptase. Although the conformational change is at a distance from its active site, it disrupts its catalytic activity. It blocks both RNA-dependent and DNA-dependent DNA polymerase activities but does not affect the activity of the template or nucleoside triphosphate. Nevirapine does not inhibit HIV-2 reverse transcriptase or human DNA polymerases α, β, or γ. The resistance to the drug results from site-directed mutagenesis at codons 103 or 181 but also at 100, 106, 108, 188, and 190 of viral reverse transcriptase. The development of resistance to one non-nucleoside reverse transcriptase implies that HIV will also be resistant to the rest of the drugs in this class.

After oral administration, nevirapine is rapidly absorbed with a bioavailability of 93 %, and peak plasma concentrations are achieved in 4 h. Food or antacids do not interfere with its absorption. It is very lipophilic, it crosses the placenta, and its presence has been reported in breast milk. Nevirapine is mainly metabolized by cytochrome P450 system (CYP3A4 and CYP2B6) to hydroxylated metabolites, and after metabolism the primary route of excretion is through urine. It has an elimination half-life of 25–30 h. Nevirapine can induce its own metabolism by stimulating the cytochrome P450 system, which results in the reduction of the half-life of the subsequent doses. In combination with other anti-retroviral agents, nevirapine is recommended for the treatment of HIV infection in adults and children. It should not be administered alone, since resistance develops rapidly.

Side Effects and Drug Interactions

The adverse effects associated with the use of nevirapine include rash, mild macular or popular eruptions, pruritus, elevated hepatic transaminases, and hypersensitivity reaction. There is also a risk of hepatitis/hepatic failure that may be associated with muscle ache, fatigue, malaise, and/or renal dysfunction. The use of nevirapine is rarely associated with Stevens–Johnson syndrome but is potentially fatal.

Since nevirapine is metabolized by the cytochrome P450 system and induces 3A4 and 2B6, the other drugs, which are also metabolized by these isoenzymes, will have low plasma levels when given in combination. It also increases the clearance of methadone and results in methadone withdrawal. Nevirapine decreases the plasma concentrations of norethindrone, ethinyl estradiol, and protease inhibitors (HIV).

9.4.2.4 Delavirdine

Delavirdine is a synthetic non-nucleoside reverse transcriptase inhibitor that after directly binding to HIV-1 reverse transcriptase, blocks RNA-dependent and DNA-dependent DNA polymerase activities. It disrupts the catalytic activity of the enzyme, after causing a conformational change in HIV-1 reverse transcriptase, and does not compete with deoxynucleoside triphosphates. The resistance to delavirdine results from site-directed mutagenesis at codons 103 or 181,106, 188, and 236 of viral reverse transcriptase. As with nevirapine, the developed resistance will be applicable to the entire class of these drugs (non-nucleoside reverse transcriptase inhibitors).

After oral administration, delavirdine is rapidly absorbed with a bioavailability of 85 %; peak plasma concentrations are achieved in 1 h. It may be administered with or without food, and its absorption may be inhibited by proton pump inhibitors, H2 receptor antagonists, as well as achlorhydria. Delavirdine is metabolized by cytochrome P450 system (CYP3A4), and the major metabolic pathways are N-dealkylation and pyridine hydroxylation. Ninety-eight percent of the drug is bound to plasma albumin, and its elimination half-life is about 6 h, although considerable variability in different patients has been noted. Delavirdine inhibits its own metabolism by inhibiting CYP3A4 activity. In combination with at least two other anti-retroviral agents, delavirdine is recommended for the treatment of HIV infection. However, it is not widely used because of its short half-life.

Side Effects and Drug Interactions

The most common side effects associated with the administration of delavirdine are macular, popular, and erythematous pruritic rashes, involving trunk and extremities as well as severe dermatitis. Fatal hepatitis is not associated with its use, but elevated hepatic transaminases have been reported. Other rare side effects of delavirdine include Stevens–Johnson syndrome and neutropenia.

Delavirdine should not be used in combination with drugs, which are CYP3A4 substrates such as pimozide, midazolam, triazolam, amiodarone, propafenone, and ergot derivatives. The inducers of hepatic P450 system, rifampin, rifabutin, phenobarbital, phenytoin, or carbamazepine should not be used in combination with delavirdine. It also increases the plasma levels of HIV protease inhibitors.

9.4.2.5 Etravirine (Intelence)

Etravirine is a diarylpyrimidine-based second-generation non-nucleoside reverse transcriptase inhibitor that is active against multiple subtypes of HIV-1 and viruses, which are resistance to first-generation non-nucleoside reverse transcriptase inhibitors. It can bind to the enzyme in a variety of conformations. In combination with nucleoside reverse transcriptase inhibitors and protease and/or integrase inhibitors, excellent efficacy is observed. The drug decreases HIV titer and increases the number of $CD4^+$ cells. The viral resistance to other current non-nucleoside reverse transcriptase inhibitors is not conferred to etravirine. It is active against strains, which are resistant to efavirenz (K103N) and nevirapine (Y181C). Nonetheless, the resistance will eventually develop. A number of drug-resistant-associated mutations have been identified for this dug. The patients with galactose intolerance should not take this drug because it contains galactose. The side effects include Stevens–Johnson syndrome, erythema multiforme, toxic epidermal necrolysis, hypersensitivity reactions, rash, and in some rare cases hepatic and other failures.

9.4.2.6 Rilpivirine

Rilpivirine is a non-nucleoside reverse transcriptase inhibitor that was approved for non-nucleoside reverse transcriptase inhibitor-naïve patients. Its structure and binding to reverse transcriptase are similar to etravirine. This allows reorientation of both drugs in the viral enzyme. Rilpivirine is highly potent when compared with the older generation non-nucleoside reverse transcriptase inhibitors. This is speculatively attributed to its internal conformational flexibility and the plasticity of its interaction with the binding site. It is active against HIV-1 of multiple subtypes and is effective in viruses, which are resistant to other non-nucleoside reverse transcriptase inhibitors. Similar to other drugs, it is used in combination with other anti-retroviral drugs. It reduces HIV titer and increases the number of $CD4^+$ T cells. Rilpivirine eventually develops resistance, and its resistance profile is similar to etravirine. The main pharmacokinetic interactions, in reference to rilpivirine, include reduced bioavailability when given with rifampicin, rifabutin, or antacids and increased bioavailability with ketoconazole. The side effects include nausea, dizziness, nightmare, dyspepsia, rashes, somnolence, vertigo, and asthenia.

9.5 Human Immunodeficiency Virus Protease Inhibitors

In the life cycle of HIV, its RNA is translated into a polypeptide chain that is composed of several individual proteins including protease, integrase, and reverse transcriptase. However, in this form, these enzymes are not functional. They must be cleaved by viral proteases from the assembled sequence in order for them to become functional. These posttranslational modifications allow the enzymes to facilitate the production of new viruses. The protease itself is made up of two 99-amino-acid monomers, and an aspartic acid residue in the monomer is required for the cleavage. The protease inhibitors inhibit the enzyme protease and consequently interfere with viral replication and maturation, by preventing proteases from cleaving proteins into peptides. In humans these drugs inhibit HIV gag and pol polyprotein cleavage, which are part of the essential viral structural components, P7, P9, P17, and P24, and protease as well as other enzymes. As a result, the protease inhibitors interfere with a maturation of HIV virus particles.

9.5.1 Saquinavir

Saquinavir is a peptide-like substrate analog which inhibits HIV protease after binding to its active site and is active against both HIV-1 and HIV-2 maturation. It blocks splicing of the viral polyproteins, which results in the production of immature viral particles that lack the ability to infect other cells. The resistance to saquinavir is associated with mutations in protease genes G48V and L90M, whereas secondary mutations are associated with codons 36, 46, 82, 84, 101, 154, and 184. Multiple mutations are necessary to render strong resistance to the drug.

Its bioavailability is about 4 % after a single dose (600 mg) of its hard gelatin form (Invirase) in the presence of a high-fatty meal, and this low bioavailability is attributed to incomplete absorption and first-pass metabolism. Ninety-eight percent of saquinavir is bound to plasma proteins, and its penetration into the brain is limited by P-glycoprotein transporter in the capillary endothelial cells of the blood–brain barrier. Saquinavir has a short half-life and is metabolized by intestinal and hepatic cytochrome P450 system, where more than 90 % of the hepatic metabolism is mediated by the isoenzyme CYP3A4, resulting in an inactive hydroxylated compound. The metabolites are excreted primarily through feces and biliary system, whereas the urinary excretion is minimal. Saquinavir is available as Invirase, which is its hard gelatin form, and Fortovase that is its soft gelatin form. In combination with other anti-retroviral agents, Invirase is recommended for the treatment of HIV infection, and due to its low bioavailability, considerably higher doses are recommended. The two preparations, Invirase and Fortovase, are not bioequivalent and could not be used interchangeably. While Fortovase can be used as a sole protease inhibitor in a combination therapy, Invirase could only be used in combination with ritonavir.

9.5.1.1 Side Effects and Drug Interactions

The adverse effects of saquinavir are mild, but predominantly they are related to gastrointestinal discomfort including abdominal discomfort, nausea, diarrhea, and vomiting. Lipodystrophy may result from its long-term use. Rarely, saquinavir causes confusion, weakness, ataxia, seizures, headache, and liver abnormalities.

The pharmacokinetics of saquinavir is modified by agents that alter isoenzyme CYP3A4 of the cytochrome P450 system and P-glycoprotein transporter. It should not be administered with midazolam, triazolam, and ergot derivatives. The plasma concentrations of saquinavir are lower when coadministered with efavirenz, nevirapine, or rifampin. Ritonavir reverses the effects of nevirapine on saquinavir. The coadministration of astemizole, terfenadine, amiodarone, bepridil, quinidine, propafenone, or flecainide with saquinavir is also not recommended due to potential for serious and/or life-threatening reactions.

9.5.2 Ritonavir

Ritonavir is a peptidomimetic protease inhibitor with activity against the HIV-1 and HIV-2 proteases. Following the binding of ritonavir to the viral protease, the enzyme is no longer able to process the gag–pol polyprotein precursors, resulting in the production of immature HIV particles, which lack the capability to infect other cells. The resistance to the drug results from site-directed mutagenesis of viral protease. The mutations take place at codon 82, 84, 71, or 46, but codons 20, 32, 54, 63, 84, and 90 may also be involved. Mutations at multiple codons are necessary to render strong resistance to the drug.

The peak levels of ritonavir are reached between 2 and 4 h after oral administration, and its peak levels decrease by 23 % and absorption decreases by 7 %, when compared to the fasting conditions. Ritonavir is 98–99 % bound to plasma proteins (α1-glycoprotein), and its penetration into the brain is limited by P-glycoprotein transporter in the capillary endothelial cells of the blood–brain barrier. Ritonavir is metabolized by the cytochrome P450 system isoenzyme cytochrome P450, family 3, subfamily A (CYP3A), where its major metabolite is isopropyl thiazole oxidation metabolite (M-2), which is equivalent to the parent drug in its pharmacologic activity, but has lower plasma levels, and the parent drug and its metabolites are mainly excreted in feces. In combination with other anti-retroviral agents, ritonavir is recommended for the treatment of HIV infection.

9.5.2.1 Side Effects and Drug Interactions

The adverse effects of ritonavir comprise of gastrointestinal discomfort including abdominal pain, nausea, diarrhea, vomiting, asthenia, and neurological disturbances. Taste perversion, peripheral paresthesias, and lipodystrophy including

elevated levels of triglycerides and cholesterol have also been reported with its administration.

Ritonavir increases the plasma levels of triazolam, pimozide, midazolam, ergot derivatives, propafenone, and amiodarone by delaying their elimination, since it is a very potent inhibitor of CYP3A4. Rifampin, due to its ability to induce CYP3A4, will reduce the plasma levels of ritonavir, and their coadministration is not recommended. Since ritonavir is also an inhibitor of CYP2D6, its coadministration with most antidepressants, certain antiarrhythmic, and narcotic analgesics should be carefully monitored.

9.5.3 Indinavir

Indinavir is a peptidomimetic hydroxyethylene protease inhibitor that is ten times more potent against HIV-1 enzyme than HIV-2. Following the binding of indinavir to the viral protease, the protease is no longer able to process the gag–pol polyprotein precursors, resulting in the production of immature HIV particles that lack the capability to infect other cells. The resistance to the drug develops from site-directed mutagenesis at codons 46, 82, and 84 of viral protease. Furthermore, codons 10, 20, 24, 46, 54, 63, 71, 82, 84, and 90 may also be involved. Resistance results from multiple and variable substitutions at these codons.

Indinavir is absorbed rapidly in fasting state, and peak plasma concentrations are reached in 0.8 h. A meal high in fat and protein calories causes a 77 % decrease in plasma concentrations of indinavir but a light meal does not affect its plasma concentration. Sixty percent of indinavir is bound to plasma proteins, and its half-life is 1.8 h. Indinavir is metabolized by cytochrome P450 system (CYP3A4) to a glucuronide conjugate and other oxidative metabolites, and most of the drug and its metabolites are excreted in feces. The problems of its short half-life and interference by food could be addressed when it is coadministered with ritonavir. In combination with other anti-retroviral agents, indinavir is indicated for the treatment of HIV infection.

9.5.3.1 Side Effects and Drug Interactions

Some patients receiving indinavir exhibit nephrolithiasis/urolithiasis including flank pain, which may be accompanied with or without hematuria. The frequency of nephrolithiasis is dependent on the period of treatment with indinavir. Other side effects associated with indinavir include insulin resistance, hyperglycemia, asymptomatic hyperbilirubinemia, HIV lipodystrophy syndrome, and skin abnormalities. Indinavir should not be coadministered with drugs that affect cytochrome P450 system (CYP3A4). Antacids are not recommended within 2 h of its administration, specifically didanosine containing an antacid buffer.

9.5.4 *Nelfinavir*

Nelfinavir is a non-peptide protease inhibitor that is active against both HIV-1 and HIV-2. Following the binding of nelfinavir to the HIV protease, the protease is no longer able to process the gag–pol polyprotein precursors, resulting in the production of immature HIV particles that lack the capability to infect other cells. The resistance to the drug results from site-directed mutagenesis of viral protease. The high level of resistance is associated with codon 30. Other viral protease mutations have been reported at codons 35, 46, 71, 77, and 88.

The peak levels of nelfinavir are reached between 2 and 4 h after oral administration, and the content of fat in food affects its absorption. The drug is more than 98 % protein bound with a half-life in plasma between 3.5 and 5 h. Its penetration into the brain is limited by P-glycoprotein transporter in the capillary endothelial cells of the blood–brain barrier. After a single oral dose of 750 mg, 82–86 % of the drug remains unchanged. The rest is metabolized by cytochrome P450 system, primarily by isoenzyme CYP2C19, and some by isoenzymes CYP34A and CYP2D6. This results in one major and several minor oxidative metabolites, all of which along with the parent drug are excreted in feces. In combination with other anti-retroviral agents, nelfinavir is indicated for the treatment of HIV infection. It is used in children and pregnant women due to its relatively mild side effects.

9.5.4.1 Side Effects and Drug Interactions

The most common side effect associated with the use of nelfinavir is diarrhea. Other less frequent adverse effects include elevated triglycerides and cholesterol plasma levels, nausea, rash, as well as hyperglycemia.

The drugs that inhibit CYP3A will increase the plasma concentrations of nelfinavir, and the drugs, which induce CYP3A or CYP2C19 will reduce its plasma concentration and its therapeutic effects. Its coadministration with drugs that are metabolized by CYP3A will increase the plasma concentration of other drugs, which could augment its adverse effects. Nelfinavir also reduces the levels of ethinyl estradiol and norethindrone by inducing hepatic drug metabolizing enzymes and zidovudine, by induction of glucuronyl S-transferase. No clinically significant drug interactions have been reported with most of the reverse transcriptase inhibitors.

9.5.5 *Lopinavir*

Lopinavir is an inhibitor of HIV protease, with a structure similar to ritonavir, and is active against both HIV-1 and HIV-2. Following the binding of lopinavir to the HIV protease, the protease is no longer able to process the gag–pol polyprotein precursors, resulting in the formation of immature HIV particles, which lack the capability to infect other cells. The resistance to the drug ultimately results from

site-directed mutagenesis at codons 10, 20, 24, 36, 46, 53, and/or 73 of viral protease. This drug is only available in a co-formulation with ritonavir, which helps increase the plasma levels of lopinavir.

Due to first-pass metabolism, if administered alone, lopinavir has varied plasma levels. This problem is overcome by adding ritonavir to the formulation. Its oral absorption is rapid, and its bioavailability is increased by food rich in fat content. Lopinavir is metabolized by cytochrome P450 isoenzyme CYP3A4. Most of the drug in the plasma is bound to α1-glycoprotein. In combination with other anti-retroviral agents, lopinavir is indicated for the treatment of HIV infection.

9.5.5.1 Side Effects and Drug Interactions

The adverse effects of the administration of this co-formulation include gastrointestinal problems such as nausea, diarrhea, vomiting, loose stool, increased plasma cholesterol, and triglyceride levels.

St. John's wart, rifampin, efavirenz, nevirapine, and amprenavir will lower plasma concentrations of lopinavir due to their effect on cytochrome P450 enzyme CYP3A4. Lopinavir increases plasma concentrations of ergot derivatives, triazolam, midazolam, and propafenone and should not be given together.

9.5.6 Amprenavir and Fosamprenavir

Amprenavir is a non-peptide protease inhibitor that is active against both HIV-1 and HIV-2; fosamprenavir is the prodrug for amprenavir and has better bioavailability. After binding to the active site of the viral protease, it inhibits the processing of viral gag and gag–pol polyprotein precursors, resulting in the production of immature HIV particles, which lack the capability to infect other cells. The resistance to the drug results from site-directed mutagenesis primarily at codons 50 and 84 and also at codons 10, 32, 46, 54, and 90.

The oral absorption of amprenavir is rapid, and peak concentrations are reached between 1 and 2 h, and administration of both amprenavir and fosamprenavir with food is not a concern. Fosamprenavir is dephosphorylated to amprenavir in intestinal mucosa. Amprenavir is 90 % bound to plasma protein with most to α1-glycoprotein. It is metabolized by the cytochrome P450 system, CYP3A4, in the liver, and more than 90 % of the drug is excreted after its metabolism in feces. In combination with other anti-retroviral agents, amprenavir/fosamprenavir is indicated for the treatment of HIV infection.

9.5.6.1 Side Effects and Drug Interactions

The adverse effects associated with amprenavir comprise of gastrointestinal symptoms including nausea, diarrhea, and vomiting; other side effects include headache, fatigue, and skin eruptions.

The drugs that induce cytochrome P450 system isoenzyme CYP34A will inhibit the blood levels of amprenavir. It decreases the plasma levels of methadone and delavirdine and increases the plasma levels of rifabutin, ketoconazole, and atorvastatin. This may be the result of the ability of amprenavir to inhibit as well as induce cytochrome P450 system isoenzyme CYP34A.

9.5.7 Tipranavir

Tipranavir is a non-peptide protease inhibitor that is active against HIV-1. Following the binding of tipranavir to the HIV protease, the protease is no longer able to process the gag–pol polyprotein precursors, resulting in the production of immature HIV particles that lack the ability to infect other cells. The resistance to the drug results from site-directed mutagenesis at codons 10, 13, 33, 36, 45, 71, 82, and 84 of viral protease.

The oral absorption of tipranavir is limited, and the bioavailability is increased with a high-fat meal. Its binding to plasma proteins is more than 99.9 % where it binds to both α_1-glycoprotein and albumin. Tipranavir is metabolized by cytochrome P450 isoenzyme CYP3A4. Ritonavir decreases its first-pass clearance, and most of the drug is excreted in the feces. In combination with other anti-retroviral agents, ritonavir is indicated for HIV-infected patients.

9.5.7.1 Side Effects and Drug Interactions

The adverse effects after its coadministration with ritonavir include diarrhea, nausea, vomiting, and bronchitis. Tipranavir induces P-glycoprotein, resulting in a number of drug–drug interactions. When administered in combination with ritonavir, it inhibits CYP3A and consequently will increase the plasma levels of the drugs metabolized by cytochrome P450 isoenzyme CYP3A. Its coadministration with amiodarone, quinidine, propafenone, flecainide, bepridil, terfenadine, and astemizole is not recommended as this combination could cause potential life-threatening cardiac arrhythmias. Other drugs including ergot derivatives, rifampin, and cisapride also are contraindicated in combination with tipranavir.

9.5.8 Atazanavir

Atazanavir is an azapeptide protease inhibitor. It is active against HIV-1. Following the binding of atazanavir to the HIV protease, the protease is no longer able to process the gag–pol polyprotein precursors. This results in the production of immature

HIV particles, which lack the capability to infect other cells. The resistance to the drug results from site-directed mutagenesis of viral protease. The resistance is associated with codon 50. In combination with other anti-retroviral agents, atazanavir is indicated for the treatment of HIV infection.

Its oral absorption is rapid, and the peak levels are reached in about 2.5 h. Food affects its absorption with a 70 % increase after a light meal and a 30 % after a meal rich in fat content. Atazanavir is 86 % bound to plasma proteins including both α_1-glycoproteins and albumin. Food also helps overcome the interindividual pharmacokinetic variability associated with this drug. Atazanavir is extensively metabolized through mono-oxygenation and deoxygenation. The cytochrome P450 isoenzyme CYP3A4 is involved in the metabolism. Most of the metabolites are excreted in feces with some in urine.

9.5.8.1 Side Effects and Drug Interactions

The adverse effects of atazanavir include fever, jaundice/scleral icterus, myalgia, and diarrhea. Its coadministration is not recommended with the drugs that induce cytochrome P450 isoenzyme CYP3A4. Ritonavir increases plasma concentrations of atazanavir. It is an inhibitor of cytochrome P450 isoenzymes CYP3A4, CYP2C8, and UGT1A1. The coadministration of atazanavir with calcium channel blockers, HMG-CoA reductase inhibitors, immunosuppressants, and phosphodiesterase 5 inhibitors should be carefully monitored.

9.5.9 Darunavir

Darunavir is a non-peptide second-generation protease inhibitor of HIV-1. In combination with ritonavir, it is superior to lopinavir plus ritonavir. Darunavir is a selective inhibitor of the cleavage of HIV-encoded Gag–Pol polyproteins in infected cells resulting in the formation of immature virions. It is not known to inhibit the activity of other protease, nucleoside, and non-nucleoside reverse transcriptase inhibitors. Darunavir has a half-life of 15 h and is metabolized by cytochrome P450 3A (CYP3A), and therefore its levels are greatly increased since it is required to be coadministered with ritonavir. It does not induce P-glycoprotein that is the case with tipranavir, and as a consequence, it has fewer drug–drug interactions. Darunavir is indicated for the treatment of patients who are infected with strains, which are resistant to more than one protease inhibitor. The side effects associated with the use of darunavir include nausea, diarrhea, moderate increase in the levels of lipids and transaminases, mild to moderate rash, headache, abdominal pain, and vomiting. Severe rashes are rare but have been reported.

9.6 HIV Integrase Strand Transfer Inhibitors

9.6.1 Dolutegravir

Dolutegravir is an HIV integrase strand transfer inhibitor used for the treatment of HIV infection. The drug is given to the broad population of HIV-infected patients. It can be administered in patients who have not been treated with anti-retroviral drugs (treatment-naïve) and HIV-infected adults who have previously taken anti-retroviral drugs (treatment experienced). The drug can also be given to the patients who have previously taken integrase inhibitors. The children who are at least 12 years old and weigh a minimum of 90 lb can also take this drug, whether they are treatment naïve or treatment experienced but have not previously administered other integrase inhibitors. The side effects of dolutegravir include allergic reactions, headache, insomnia, and abnormal liver function in hepatitis B- and C-infected patients.

9.6.2 Elvitegravir

Elvitegravir is an HIV integrase strand transfer inhibitor used for the treatment of HIV infection. The drug is used in adult patients beginning HIV treatment for the first time. It is given as a component of a fixed dose combination called Stribild, which is composed of elvitegravir, emtricitabine, and tenofovir. The patients taking once-daily elvitegravir in combination with ritonavir had significantly higher decrease in viral load compared with patients administered ritonavir-boosted protease inhibitor. Its side effects are few but include diarrhea and rash.

9.6.3 Raltegravir

Raltegravir is an HIV integrase strand transfer inhibitor used for the treatment of HIV infection. It changes HIV viral decay and dynamics, significantly. Raltegravir is used in all patients. Due to mutagenesis of HIV, it is administered in combination with other anti-retroviral drugs. It is well tolerated by the patients. The side effects include flu-like symptoms, weakness, dizziness, bruising, muscle pain, increased risk of infection, pale skin, and gastrointestinal disorders. It does not increase serum levels of cholesterol or triglycerides. Simultaneous use of statins, phenobarbital, or rifampin may cause drug–drug interactions.

9.7 Drugs Inhibiting Viral Binding

9.7.1 Enfuvirtide

Enfuvirtide, a biomimetic peptide with a sequence matching the gp41 receptor on HIV membrane, blocks the entry of HIV into the host cell by inhibiting membrane fusion that allows the entry of the virus into $CD4^+$ cells. It is active only against HIV-1, and the resistance develops as a result of mutation in the drug-binding region of gp41.

Enfuvirtide requires parenteral administration, and the peak levels are reached in about 4 h. Its bioavailability is 84 %, and the drug is 92 % protein bound in plasma. Its half-life is 3.8 h, and enfuvirtide is metabolized in the liver, which is not at significant levels. Enfuvirtide is approved for the treatment of HIV in patients whose response to anti-retroviral therapeutic regimen is not satisfactory, and there is an indication that HIV is still replicating.

9.7.1.1 Side Effects and Drug Interactions

Irritation at the site of injection is a common problem that may include pain, swelling, redness, induration, nodules, and cysts at the site of injection. No drug interactions are known.

9.8 Chemokine Receptor Antagonists

9.8.1 Maraviroc

Maraviroc is a chemokine C–C motif 5 (CC5) receptor antagonist. CCR5 is required for the entry of most HIV strains into $CD4^+$ T cells and macrophages. Maraviroc binds to CCR5, blocking the association of GP120 with the chemokine receptor. This blocks the entry of HIV into $CD4^+$ T cells and macrophages. Tropism testing is necessary before this dug is used to treat HIV infection, to make certain that HIV is not using the other co-receptor CXCR4 to enter the cells. It is indicated for patients infected with only CCR5-tropic HIV-1, in combination with other anti-retroviral drugs. Maraviroc has shown benefit in patients who have or have not been previously treated.

High-fatty food results in a reduction of plasma concentrations of maraviroc as compared with fasting. Seventy-six percent of the drug is bound to human plasma proteins. It has moderate affinity for both albumin and α1-glycoprotein. The drug is mostly present in the plasma. Higher levels have been noticed in vaginal fluid and

vaginal tissue biopsies. Most of the drug while in the plasma remains unchanged, while the rest undergo oxidation and N-dealkylation, with no pharmacologic effects. It is metabolized by CYP3A4. Only 23 % of the drug is excreted by renal system; the rest is cleared metabolically. No other enzymes of cytochrome P450 system are involved in its metabolism. The drug that modulates the function of CYP3A4 affects the profile of maraviroc. The side effects of maraviroc include dizziness, low fever, itching, nausea, loss of appetite, jaundice, upper stomach pain, and dark urine.

9.9 Booster Drug

9.9.1 Cobicistat

Cobicistat is used in patients infected with HIV due to its ability to inhibit liver enzymes that metabolize other anti-HIV drugs. It is a potent inhibitor of cytochrome P450 3A enzymes, including the CYP3A4 subtype and 2D6 (CYP2D6) enzymes as well as an inhibitor of cellular P-glycoprotein transporters, BCRP, OATP1B1, and OATP1B3. Cobicistat also suppresses intestinal transport proteins, resulting in increased absorption of several anti-HIV drugs. It has no anti-HIV activity of its own but is used as a booster. Its side effects include nausea, diarrhea, and fatigue. Cobicistat suppresses renal tubular secretion of creatinine and enhances serum creatinine levels; as a consequence, there is a decrease in estimated glomerular filtration rate (GFR) without its true decline. It is metabolized by CYP3A and CYP2D6; as a result drugs that induce or inhibit the action of this isoenzyme may alter its serum concentrations. Azole antifungals and clarithromycin may increase serum cobicistat concentrations. Rifabutin and some antiepileptic drugs (carbamazepine and phenytoin) may decrease its levels.

A combination of cobicistat with HIV integrase strand transfer inhibitor, elvitegravir, results in higher concentration of the drug in the body even with lower doses and as a consequence limiting its side effects. Stribild is a combination of elvitegravir/emtricitabine/tenofovir/cobicistat. Prezcobix contains protease inhibitor darunavir with cobicistat, and Evotaz contains Reyataz (atazanavir), a protease inhibitor, with cobicistat. Both Evotaz and Prezcobix are used in combination with other antiretroviral agents for the treatment-naïve and treatment-experienced HIV-1-infected adults (Table 9.1).

Table 9.1 Future potential target mechanisms of anti-HIV therapy

Mechanisms
Inhibitors of transcription provirus
Inhibitors of translation of viral mRNA (antisense oligonucleotides)
Inhibitors of posttranslational glycosylation

9.10 Combination Therapy for AIDS

The Department of Health and Human Services has published guidelines for the use of anti-retroviral agents in HIV-infected adults and adolescents in April 2015. The panel confirmed that the regimens that demonstrated the best results included either integrase strand transfer inhibitor-based regimens or protease inhibitor-based regimens. The panel suggested that the choice of an anti-retroviral regimen should be individualized depending on the patient and the specific pharmacologic effects of the drug. The latest recommendation of the panel for naïve patients is shown in Table 9.2.

9.11 Vaccines for HIV Infection

The acquired immune deficiency syndrome (AIDS) does not fit the paradigm for classical vaccines for a number of reasons. The classical preventive vaccines enhance natural immunity against microbes that change a little or none at all, whereas HIV mutates. There is some level of initial replication and dispersal at the point of entry before the virus reaches its target tissue, which allows the immune system to mount a response and possibly eliminate it. This response leaves some memory T cells, and an individual recovers from the infection and reinfection, or prior immunization produces a robust response to prevent the disease, but there are no recovered HIV patients. Most vaccines are whole-killed or live-attenuated organisms, but killed HIV does not retain antigenicity. In response to HIV initially, there is an increase in the numbers of HIV-specific $CD8^+$ and $CD4^+$ T cells, which reduce the virus levels. Furthermore, the binding antibodies are produced in about 6–12 weeks after infection, and the neutralizing antibodies are not secreted until after a reduction in virus levels due to the production of $CD8^+$ cells; however, at this stage, there are rapid genetic mutations in the virus envelope which greatly reduces the neutralizing effects of the antibodies. Another challenge that HIV possesses is

Table 9.2 Suggested combination therapy for anti-retroviral-naïve patients

Integrase strand transfer-based regimens
Dolutegravir/abacavir/lamivudine[a]
Dolutegravir plus tenofovir/emtricitabine
Elvitegravir/cobicistat/tenofovir/emtricitabine[b]
Raltegravir plus tenofovir/emtricitabine
Protease inhibitor-based regimen
Darunavir/ritonavir plus tenofovir/emtricitabine[c]

Source: DHHS April 2015
[a]Only for patients who are HLA-B5701 negative
[b]Only for patients with pre-anti-retroviral therapy
[c]Lamivudine may be substituted for emtricitabine and vice versa. CrCl >70 mL/min

its ability to establish pools of latently infected resting $CD4^+$ T cells during very early stages of infection, resulting in an indefinite infection which takes the ability of the vaccine to eradicate the virus away, and as a consequence, virtually no person clears HIV infection. Other viral infections do not follow this path where initial viral replication does not result in the establishment of permanent viral reservoirs. Lastly, a lack of suitable animal models to test HIV vaccines continues to be a challenge.

Nonetheless, scores of strategies had been employed since the identification of HIV, despite its enormous genetic diversity and unique features. Figure 9.3 shows some of these strategies. A critical problem is the fact that the structures of the HIV's outer envelope and monomeric GP120 are different, and the outer membranes of circulating HIV hide its epitope so that genetically engineered GP120 is unable to mimic the response. Some of the immunogens used in an attempt to pro-

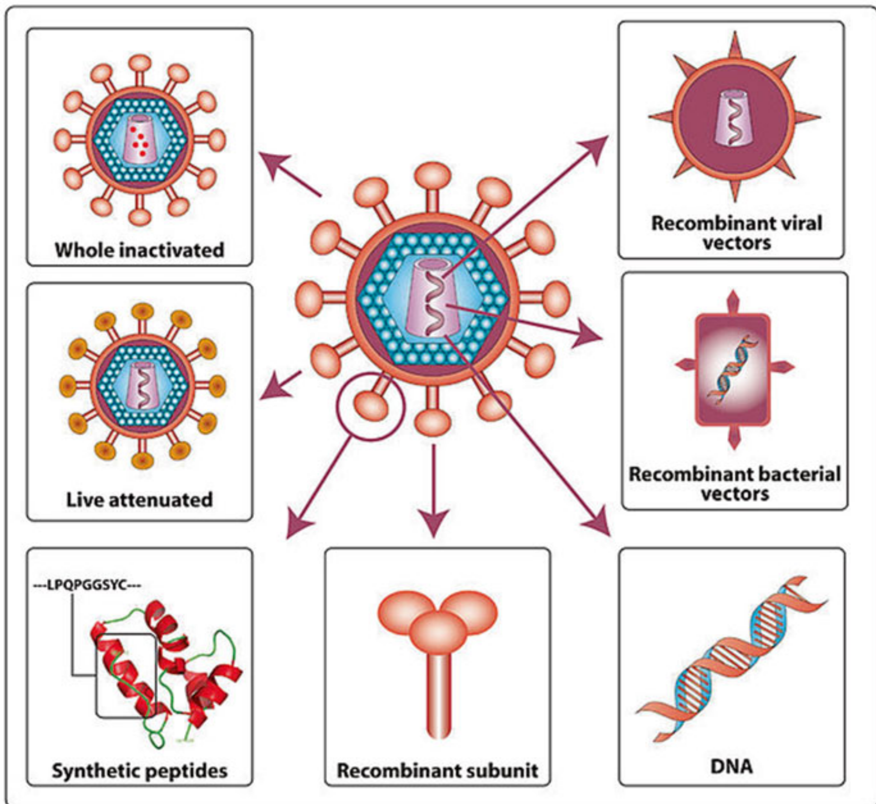

Fig. 9.3 HIV vaccine development: this figure depicts various approaches that are under investigation for the development of an HIV vaccine (Source: Wikimedia Commons. Creative Commons Attribution 2.0 Generic. Gorry PR et al. (2007) Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. *Retrovirology*. 4: 66)

duce broadly neutralizing antibodies included whole-killed HIV, pseudovirions, live vector viruses (non-HIV viruses engineered to carry gene-encoding HIV proteins), naked DNA containing one or more HIV genes, HIV peptides, and most notably viral surface proteins such as GP120 but with limited success. Since the identification of GP120 on the envelope of the virus as the binding site for CD4, which allows the attachment and entry of the virus into human cells, major effort has been devoted to develop a vaccine, which contains genetically engineered GP120 and the larger glycoprotein GP160 to inhibit the infection, but this approach failed to provide protection against HIV infection. More recently, emphasis has been placed on using novel immunogen developed by modifying viral envelope which may induce broadly neutralizing antibodies. The goals of some of these strategies are to mimic native trimer on virion surface, redirect immune responses to conserved conformational epitopes, and redirect responses away from variable epitopes.

As opposed to the development of vaccines that produce antibodies, the development of T-cell vaccines is an alternate concept. These vaccines are designed to induce primarily T-cell responses, which will control the viral proliferation and viral levels during the early stages of infection and will delay the disease progression. These vaccines will not prevent infection but inhibit HIV levels and protect uninfected memory $CD4^+$ T cells. It is expected that HIV-infected patients receiving this vaccine may remain disease-free for a prolonged period of time. A number of HIV vaccine trials have focused on $CD8^+$ cell-mediated products that employ either viral vectors alone or in combination with DNA plasmids that contain viral genes. For example, a canarypox vector vaccine in combination with a GP120, which boosts for both internal and envelope HIV proteins, has been tested. Another vaccine that contains altered adenovirus type 5, which cannot replicate but transmits HIV's gag, pol, and nef genes, has also undergone testing. A different approach is a two-step design where a vaccine made up of naked DNA is first administered to prime an immune response against both the internal and external HIV proteins followed by a booster shot of inactivated adenovirus vector that may induce specific responses to HIV envelope proteins and internal proteins.

At present no effective HIV vaccine is available, but many clinical trials have been performed. Studies using monoclonal antibodies have suggested that human can effectively respond against the virus that forms the basis that a vaccine is possible. One clinical trial that began in Thailand in 2003 reported some positive results in 2009. In this trial two vaccines, which were not successful individually, ALVAC–HIV (vCP1521) and AIDSVAX B/E (gp120), were combined. ALVAC–HIV (vCP151) is made up of a viral vector that contains three HIV genes (env, gag, and pol), which are genetically engineered. The ALVAC vector is a modified form of canarypox virus. AIDSVAX B/E is made up of gp120, which is genetically engineered. This clinical trial included 16,395 individuals who were not infected with the HIV virus. Of this group 8197 individuals received the combined vaccines, and 8198 were administered a placebo. Each individual was monitored every 6 months for 3 years to detect HIV infection. After 3 years the HIV infection rate was reduced 30 % in the vaccine-treated group as opposed to the placebo group. But this number was revised to 26 % because seven individuals had HIV infection, when the vaccine

Table 9.3 Some AIDS vaccines under development

Vaccine	Composition
Canarypox + envelope	Gag, pro, env (E + gp120 (B, E)
Adenovirus type 5	Gag, pol, nef (B)
Naked DNA followed by adenovirus type 5	Gag, pol, nef (B) Env (A, B, C) + gag, pol (B), env (A, B, C)
Canarypox + lipopeptides	Gag, pol, nef, env (B) + $CD8^+$ cells epitopes (B)
DNA polylactide co-glycolide + envelope	Gag, env + gp140(B)
DNA + modified vaccinia Ankara	Gag, pol, nef, tat, env (C)
DNA + peptides	Gag (B) many T-cell epitopes ± IL-12 or IL-15 or GM-CSF
Fowl pox + modified vaccinia Ankara	Env, gap, nef, pol, rev, tat (B)

was administered. Additional data analysis pointed out that the vaccine recipients who formed IgG antibodies to the V2 loop of the HIV outer envelope had a 43 % less chance to get infected with HIV than the patients who did not produce this specific IgG. Furthermore, a 54 % higher risk of HIV infection was associated with IgA production, as compared to those who did not synthesize antibodies. The HIV isolated from individuals who were administered with the vaccine exhibited mutations in the V2 region. In animal models, antibodies against this region produce more resistance to SIV. This makes production of IgG antibodies against the V2 loop a tempting target for future vaccine development.

In a clinical trial, a killed-whole HIV vaccine has shown some success, although previously noted that killed HIV antigen does not retain antigenicity. For the first time, a dead version of HIV was used for a vaccine. There was an increase in HIV-specific antibodies without any noticeable adverse side effects. After vaccination there was an increase of 8- and 64-fold production of antibodies, against gp120 surface antigen and p24 capsid antigen, respectively. Other approaches include the use of live-attenuated HIV-1 vaccine by employing a genetically modified HIV virus. This involves intervention in the virus codons and using an unnatural amino acid for translation. In this case, the virus would not be able to replicate due to the non-self-nature of this amino acid to the human body. A list of some AIDS vaccine under consideration is shown in Table 9.3.

While none of the vaccines will be able to eradicate the disease, however, it is expected that they will be able to prolong the disease-free period by reducing the viral levels and, as a consequence, will also reduce transmission.

9.12 Gene Editing

The site-specific modification of the human genome is called gene editing. CCR5 is an ideal receptor for gene editing in individuals infected with HIV. Its disruption is expected to increase the survival of $CD4^+$ T lymphocytes. HIV does not infect

individuals who are homozygous for delta 32/delta 32 deletion in CCR5. In a study of 12 patients with chronic aviremic HIV infection in two cohorts who were receiving highly active anti-retroviral therapy, patients were infused with autologous CD T cells with gene-edited CCR5. The gene editing was performed by using the zinc finger nucleases (ZFNs). The potential beneficial effect of this treatment includes an increase in the number of CD4+ T lymphocytes. Furthermore, the modified cells are not immunogenic as they have a long-term survival rate. The goal of the study is to repopulate CCR5-deficient central memory T cells.

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Chapter 10
Regulatory T Cells and Disease State

Abstract The regulatory T cells are a subset of T cells which suppress or regulate the immune response, protect from autoimmune disease, and maintain tolerance to self-antigens. This chapter describes the nomenclature and function of various types of Treg cells that possess immunosuppressive function. Their major subsets include natural Treg cells, peripheral Treg cells, Tr1 cells, TH3 cells, CD8 Treg cells, Qa-1-restricted $CD8^+$, $CD8^+$ $CD28^-$, and NKT cells. The factors involved in the mechanism of action of each subset are discussed. Finally, the potential of Treg cells as therapeutic targets for diseases including allergic disease, autoimmune diseases, inflammatory diseases, infections, cancer, transplant rejection, and amyotrophic lateral sclerosis is described. The treatment of patients with antigen-specific Treg cells would be an interesting approach if the issue of their propagation at a mass scale is resolved.

Keywords CD25 • CTLA-4 • CD40L • Treg • Self-tolerance • CD^+CD25^+ T cells • Tr1 cells • TH_3 cells • $CD8^+CD28^-$ T cells • $CD8^+CD122^+$ T cells • Qa-1-restricted $CD8^+$ T cells • γ/δ T cells and NKT cells • Perforin • TGF-β • Lag3 • Granzyme B-dependent killing • IL-10 • IL-12 • FoxP-3 • TGF-β • Polyendocrinopathy candidiasis ectodermal dystrophy • Thymic hypoplasia • T-cell receptor excision circles • $CD45RB^{Low}$ • CD62L • CD103 • CD152 • α€ β7 • Integrin • GITR • JAK1 • JAK3 • PTEN • PI3K • Peripheral Treg cells • Dendritic cells • CSCR4/CSCL12 signals • n regulatory T cells • I regulatory T cells • NKT cell • NKG2A • NKG2B • CD94 • Including CD45Rbl0 • $CD4^+$ • $CD103^+$ • $CD4^+CD8^+$IELS • $TCR\alpha\beta^+$ $CD8^+$IELS • CD101 • CD103 • Anti-ergotypic Treg cells • Anti-idiotypic Treg cells • $TCR\gamma/\delta^+$ anti-erg T cells • T-bet • GATA-3 • RORϒt • Treg and disease • Allergic disease • Autoimmune diseases • Inflammatory diseases • Infections • Cancer • Transplantation • Cancer immunotherapy • Amyotrophic lateral sclerosis • STAT3 • STAT5

10.1 Introduction

The immune response is designed to protect human and other organisms from disease-causing agents; it also protects from detrimental responses to self. The immune system needs to be strictly regulated because of its ability to produce inflammatory mediators, killer cells, and antibodies, which are synthesized to

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eliminate the invading organisms but can also harm other normal cells. Consequently, an immune response cannot only produce autoimmunity but it is also capable of producing other diseases due to its ability to attack normal cells that are damaged mainly by the inflammatory cytokines in a collateral damage. A number of regulatory mechanisms keep these harmful effects of the immune response in check.

Immune response is triggered by antigen presentation to the TCR in context of MHC molecules, resulting in the activation and proliferation of $CD4^+$ cells and secretion of cytokines. This activation also causes $CD4^+$ T cells to express a number of cell surface receptors including CD25 (IL-2 receptor), CTLA-4, and CD40 ligand (CD40L). This growth of antigen-activated T cells was suggested to be controlled by suppressor T cells. Niels Jerne proposed that immune response may be inhibited by special lymphocytes, which pointed to the existence of suppressor T cells. The presence of such cells initially was hinted by the observations that injection of polyclonal T lymphoblasts from a parent to F_1 hybrid blocked allograft rejection in rats. Similarly, injection with myelin basic protein (MBP)-reactive cloned T-cell line abrogated autoimmune encephalomyelitis specific for this antigen. The studies of the regulation of the anti-MBP response in autoimmune encephalomyelitis provided further foundational work in identifying the regulatory T cells. This model system allowed the understanding of the recognition of TCR peptides by regulatory T cells.

Regulatory T (Treg) cells have been defined as either having suppressor or regulatory functions. The term *regulatory* has been preferred over *suppressor* because of questions about I-J-regulated suppressor T cells. Gershon initially suggested that T cells could also have a regulatory function in addition to the role of T cells as helper cells for antibody synthesis. The initial focus was on soluble factors secreted by suppressor T cells as immune regulatory agents, and some of these soluble suppressor factors were characterized as MHC restricted. However, due to a lack of a definite cell surface marker on suppressor T cells, their existence remained controversial. The concept of the presence of suppressor T cells suffered further with the discovery of TH1 and TH2 cell subsets.

Studies by Nishizuka and Sakakura led to the conclusions that the suppression of the disease was due to the actions of thymus-derived lymphocytes and the splenocytes providing protection against the disease also originated in the thymus. However, they did not attempt to isolate the suppressor factor from the thymus. Later studies by other investigators demonstrated that only a small number of Treg cells were required to stop the development of autoimmune disease in mouse models, but the number of cells required to inhibit antibody response was much higher. The splenic T cells from mice with disease were able to transfer autoimmune disease to the newborn or adult nu/nu mice. $CD4^+$ $CD8^-$ cells were later identified as the effector and suppressor T-cell population. It was found that the removal of supressor T cells from lymphoid cells will result in the disease and the re-administration of these cells will induce self-tolerance and suppression of autoimmunity. The suppressor T cells are a very small number of cells among $CD4^+$ T lymphocytes that also express CD25 antigen, and selective depletion of $CD4^+$ $CD25^+$ T cells results in multiple manifestations of autoimmune diseases. This is a result of depletion of Treg cells from either the thymus or the peripheral lymphoid tissue.

$CD4^+$ T cells mediate suppressor function independent of $CD8^+$ T cells, and these cells classified as Treg cells maintain self-tolerance and suppress responses to foreign antigens. Although various types of Treg cells have been found, the most attention has been paid to $CD4^+CD25^+$ T cells that predominantly originate in the thymus and their centralized production is referred to as "the third function of the thymus." They are mature T cells with a distinct function, and humans lacking CD^+CD25^+ T cells exhibit severe defects in controlling autoimmune response and have abnormal immune regulation, resulting in autoimmune and allergic diseases.

Based on the immunosuppressive activity, the many different types of Treg cells include natural $CD4^+$ $CD25^+$ T cells, peripheral Treg cells, IL-10-secreting Tr1 cells, TGF-β-secreting TH3 cells, $CD8^+CD28^-$ T cells, $CD8^+CD122^+$ T cells, Qa-1-restricted $CD8^+$ T cells, γ/δ T cells, and NKT cells. The production of different types of Treg cells is distinct; some are produced as a part of innate immune response while others are produced in response to an antigen as the acquired immune response develops with a selective participation of cytokines. Their function varies and includes regulation of autoimmunity, allergic responses, infection, inflammation, and transplant tolerance. The mechanisms of action of Treg cells are also diverse and range from cell–cell interaction involving CTLA-4, perforin, TGF-β, Lag3, granzyme B-dependent killing, regulation of dendritic cells, IL-10-mediated suppression, and Treg cell-mediated IL-12 consumption.

10.2 Types of Regulatory T Cells

10.2.1 Naturally Occurring Treg Cells

The Treg cells have been classified on the basis of their site of origin or mechanism of action (Table 10.1). The site of origin is for the naturally occurring Treg cells is the thymus, and the main mechanism of action is via cell–cell interaction. These Treg cells constitutively express CD4, CD25 (which is the α chain of IL-2 receptors), FoxP-3 (forkhead-winged-helix) transcription factor, and surface CD152. A defect in FoxP3 gene results in the hyperactivation of $CD4^+$ T cells. Foxp3 is also

Table 10.1 Types of Treg cells

Cell type	Phenotype
Natural Treg cells	$CD4^+$ $CD25^+$ $FoxP3^+$
Peripheral Treg cells	$CD4^+$, $CD25^{+/-}$ $FoxP3^-$/$FoxP3^+$
Tr1 cells	$CD4^+$ $CD25^{+/-}$, $FoxP3^-$ ROG
TH3 cells	$CD4^+$, $CD25^+$, $FoxP3^+$/?
CD8 regulatory cells	$CD8^+$, $CD25^+$, $FoxP3^+$
Qa-1-restricted $CD8^+$	$CD8^+$, $CD25^+$, $CD28^+$
$CD8^+$ $CD28^-$	$CD8^+$, $CD25^+$, FoxP3?
NKT	$V\alpha24^+V\beta11^+$, $CD4^+$/$CD4^-$/$CD8^+$

expressed on $CD4^+CD25^+$ peripheral T cells and $CD4^+CD8^-CD25^+$ thymocytes but is not expressed on other thymocytes, T cells, and B cells. Naturally occurring Treg cells do not require antigen exposure for their suppressive effector function; however, their generation and some of their activity may require TGF-β. A number of costimulatory signals and cytokines are also involved in the generation of Treg cells that may include B7, TNF family molecules, CD40L, PD-1, IL-2, TGF-β, or TNF-α, and these mechanisms are independent of the avidity of TCR. This results in induction of Treg cells through a genetic program with concomitant expression of CD25 and $FOXP3^+$ or negative selection of thymocytes. Natural Treg cells possess a broad T-cell receptor repertoire that has high affinity than other T cells for the MHC Class II self-peptide ligands, which have selected them positively in the thymus. They do not produce inflammatory cytokines and they inhibit the activation, proliferation, and differentiation of a number of cell types including $CD4^+$ cells, $CD8^+$ cells, B cells, NK cells, NKT cells, and dendritic cells.

If natural Treg cells cannot be generated in the thymus or have a deficient function, this results in a number of autoimmune diseases. Impaired Treg cell generation is observed in children with thymic hypoplasia resulting from 22q-2 deletion syndrome. Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) is due to mutation in a gene called transcription factor autoimmune regulator (AIRE), and important self antigens on thymic medullary epithelial cells are regulated by AIRE. In the absence of AIRE, T cells that recognize self-antigens do not undergo negative selection and consequently are not deleted. The patients suffering from rheumatoid arthritis and multiple sclerosis exhibiting a reduced number of T-cell receptor excision circles (Trec) have suppressed activity of the thymus and its output. In juvenile rheumatoid arthritis, there are reduced numbers of Trec, suggesting premature aging of the thymus. Patients with autoimmune disease have early aged thymus, which results in poor development of Treg cells and an escape of non-Treg cells with an autoreactive TCR.

Regulatory T cells exhibit different developmental stages: one group ($CD4^+CD25^+$) expresses high CD62L and CCR7 levels and inhibits inflammation after binding to antigen-draining lymph nodes; another subgroup ($CD4^+CD25^+$ or $CD4^+CD25^-$) expresses α€ β7 integrin and suppresses local immune reactions after homing to nonlymphogenic tissues at sites of inflammation. The expression of CD25 on natural Treg cells varies from none, low, intermediate, to high, suggesting a shift of expression based on the degree of injury or inflammation.

Various other cell surface markers are also expressed on $CD4^+$ Treg cells including $CD45RB^{Low}$, CD62L, CD103, CD152 (cytotoxic T-lymphocyte antigen-4 or CTLA-4), and GITR (glucocorticoid-induced TNF receptor family-related gene). The phenotype markers expressed on naturally occurring Treg cells are shown in Table 10.2. Many of these markers are associated with activated/memory cells, and it appears that naturally occurring Treg cells may be similar to memory T cells and usually are in an antigen-primed state. The Treg cells exhibit broad antigen specificity and have enhanced ability to recognize self-antigen rather than other T-cell subsets, and their production, maintenance, and function are IL-2 dependent. The development of Treg cells in the thymus is blocked if there is a defect in

Table 10.2 Phenotypic markers of naturally occurring Treg cells

CD4
CD25
CD38
CD122
CD45low
CD45RO
CTLA-4
GITR
CD62L
CD95
CD103
TLRs 4-8

Foxp3/FOXP3 gene which controls the production of these cells. They suppress a variety of immune cells involved in both the innate and acquired immune responses; IL-2 and TCR stimulation is required to express their suppressive effects on helper T cell proliferation and IL-2 production but the subsequent immune suppression is not antigen specific. Interestingly, the normal T-cell inducers do not cause the proliferation of naturally arising $CD4^{+}CD25^{+}$ Treg cells or IL-2 secretion. However, they respond to very high doses of IL-2, mature dendritic cells as antigen-presenting cells, or anti-CD28 and, as a result, proliferate and secrete IL-2.

The cell–cell interaction is the critical mechanism of suppression by the natural Treg cells where CTLA-4, GITR, and PD-110 play a role in the contact-dependent suppression. A number of molecules expressed on natural Treg cells including CTLA-4, CD80, CD86, and CD223 are inhibitory molecules. For cell–cell interaction, a competition for antigen-presenting cells and specific MHC/peptide antigenic complexes is required.

Treg cells express all three chains (α, β, γ) of the high-affinity IL-2 receptor. Signaling for IL-2 receptor is mediated through induction of JAK1 and JAK3, which results in the phosphorylation and activation of STAT3 and STAT5. The translocation of these activated transcriptional factors to the nucleus results in the functional effects mediated by IL-2. Stimulation of IL-2 receptors also results in the activation of other pathways including mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K). IL-2 regulates self-tolerance through its involvement in the development and homeostasis of $CD4^{+}CD25^{+}$ Treg cells. Treg cells have a distinct IL-2R-mediated signal transduction pathway where, while the JAK-STAT-dependent transduction pathways are not altered, downstream signaling of PI3K is not observed. This difference in transduction pathways is associated with expression of PTEN (phosphatase and tensin homolog deleted on chromosome 10) and is correlated with the hypoproliferative response of Treg cells. PTEN is a lipid phosphatase, which is a catalyst for the reverse reaction of PI3K. Consequently, PTEN negatively activates the induction of downstream signal transduction pathway. In Treg cells, the expression of PTEN is unaltered as opposed to activated

T cells where it undergoes downregulation. IL-2-induced T-cell proliferation is dependent on PI3K-mediated signal transduction. PTEN deletion allows the expansion of Treg cells in the presence of IL-2 without compromising their regulatory activity in maintaining homeostasis and self-tolerance.

IL-2 signaling directly targets the Foxp3 gene in Treg cells. This is accomplished as a result of binding between a specific site present in the first intron of the FOXP3 gene and STAT3 and STAT5 proteins. This signaling pathway is specific for $CD4^+CD25^+$ Treg cells since an IL-2-induced expression of FOXP3 gene is not seen in $CD4^+CD25^-$ cells. This lack of effect is not due to the absence of IL-2Rα because conditions which do not require the presence of IL-2Rα such as stimulation with CD3 and treatment with high concentrations of IL-2 do not alter the effects of IL-2 on Foxp3 gene expression in $CD4^+CD25^-$ cells. Consequently, IL-2 affects $CD4^+CD25^+$ Treg cells in a unique manner, which is mediated via expression of Foxp-3 gene (Fig. 10.1).

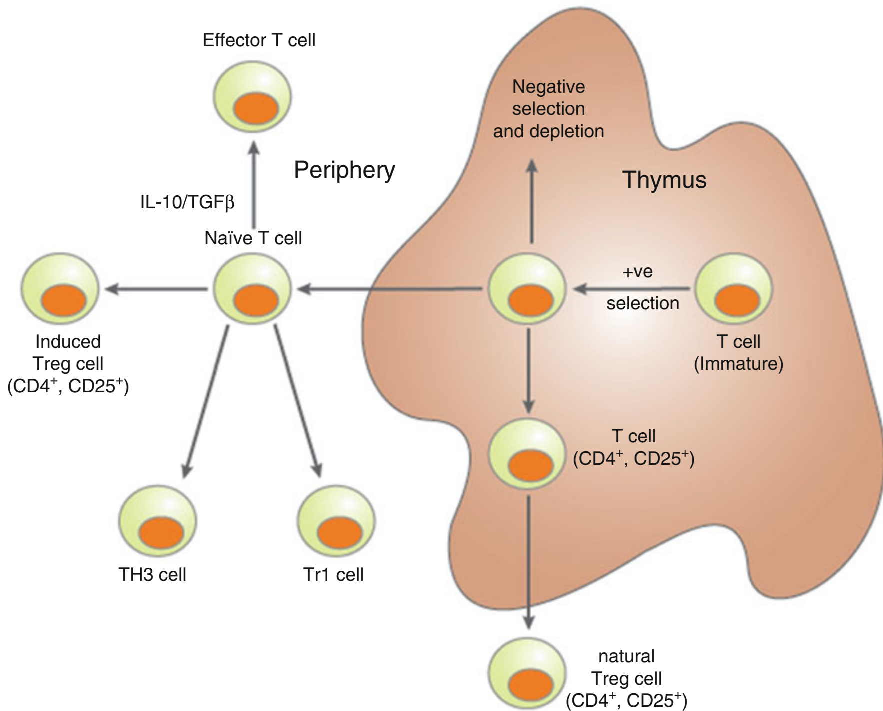


Fig. 10.1 Development of CD4+ regulatory T cells: Natural CD4+CD25+ Treg cells develop in the thymus as a result of positive selection between TCR and host antigens. The thymus-derived Treg cells are specific for antigens seen in the thymus. The autoreactive T cells undergo negative selection and are depleted by apoptosis. The acquired Treg cells develop in the periphery from naïve precursors and their specificity lie in antigens other than the ones which come in contact with the thymus. Tr1 cells are induced in the periphery when naïve T cells are exposed to an antigen in the presence of IL-10. They are not identified by a particular cell surface marker. Th3 cells are generated from naive $CD4^+$ T cells as a result of low doses of antigen via the oral route and they secrete TGF-ß

10.2.2 Peripheral (Adaptive) Treg Cells

The peripheral (adaptive) Treg cells develop in the periphery, and the stimulus for their generation is either an ongoing immune response or exposure to tolerogenic dendritic cells. Adaptive Treg cells develop from naïve precursors or mature T cells, and their specificities lie in antigens other than the ones which come in contact in the thymus, such as food antigens, pathogens, parasites, and bacterial flora. Their mechanism of action is mediated via suppressive effects of cytokines (IL-10 or TGF-β). The peripheral (adaptive) Treg cells are not generated in the thymus and are specific for both foreign antigens and self-antigens. This is in contrast to the thymus-derived Treg cells, which are specific for antigens seen in the thymus. Two models have been suggested for the generation of peripheral Treg cells. According to the linear model, after antigen recognition, naïve T cells are activated and differentiate into effector and Treg cells. Alternatively, a parallel model suggests that after activation, naïve T cells remain uncommitted and their development into effector and Treg cells is in parallel. As a result, the development of effector cells is faster than peripheral Treg cells. IL-2 is required for the development and differentiation of both types of cells. Non-Treg cells can differentiate into $CD4^+CD25^+$ Treg cells in the periphery and can function like natural Treg cells, that is, suppression of T-lymphocyte proliferation by cell–cell interaction independent of cytokines. The only main requirement for the generation of these natural Treg-like cells in periphery is activation of $CD4^+$ T cells, and their production can be achieved by exposure to oral, intravenous, or subcutaneous antigens or continued exposure to superantigen. The development of natural Treg-like cells is possible from peripheral $CD4^+CD25^+$ T cells under conditions requiring either TGF-β or both TGF-β and TCR activation, which results in $CD4^+CD25^-$ cells expressing Treg cell function and FOXP3. The treatment of human peripheral blood lymphocytes with allogeneic dendritic cells in the presence of IL-10 also results in the development of natural Treg-like cells in the periphery, as is the case with the treatment of $CD4^+CD25^-$ T cells with anti-CD3 and anti-CD28. The induction of FOXP3 is independent of TGF-β, and peripheral Treg cells look identical to natural Treg cells in phenotype, function, and gene expression. They only differ from Treg cells in their requirement for TCR and CD28 for induction. The induction of peripheral Treg cells requires immunogenic antigen exposure and the combination of an antigen directed at dendritic cells and anti-CD40 antibody. Immune response is limited against foreign antigens, and collateral damage to healthy tissue is avoided as a result of early development of FOXP3 Treg cells. The reduction in the number of effector and Treg cells may result from apoptosis and peripheral migration. However, in addition to these short-term Treg cells, long persisting Treg cells can also be induced. This induction is a result of exposure to low levels of antigen, which could be achieved without inflammation. The generation of these Treg cells may be less efficient in the beginning, but they may have prolonged presence and consequently play a role in tolerance to autoantigens.

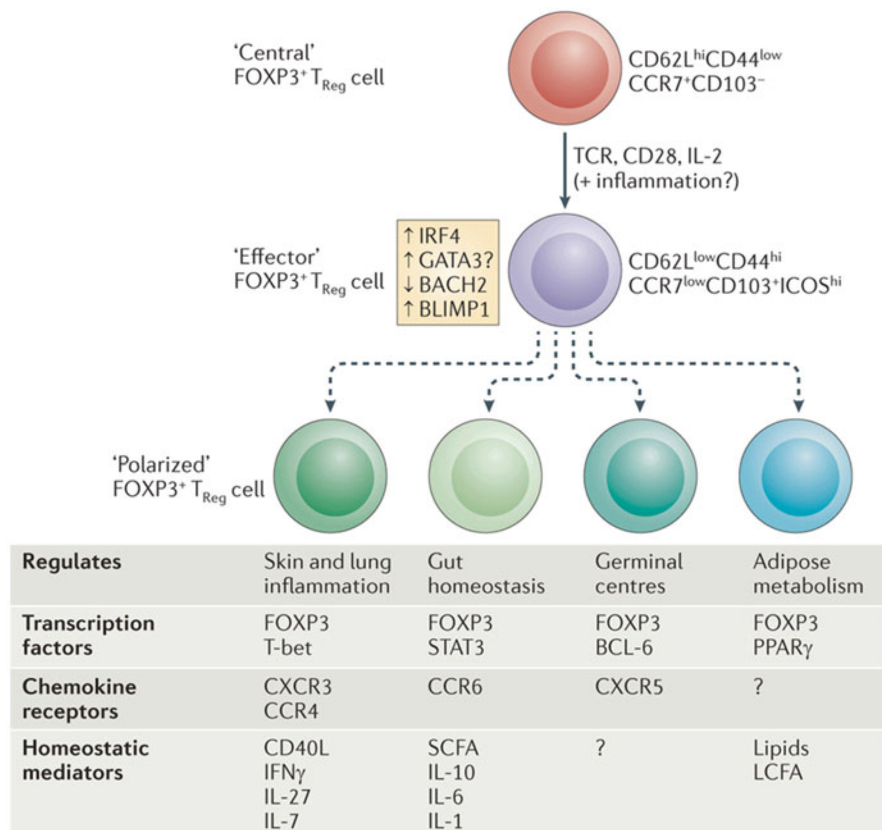
Lymphopenia promotes expansion of Treg cells. Lymphocyte activation is regulated by competition with general lymphocyte population. In the absence of such competition, both regulatory and effector cells develop sequentially as a result of weak signals. In lymphopenia the appearance of Treg cells parallel the recovery, and as soon as the accumulation of cells seizes, the Treg cells are expressed. FOXP3$^+$ cells may contribute to the development of homeostasis, and after the cell number reaches equilibrium, the generation of Treg cells begins. In periphery, generation of antigen-specific Treg cells could be rapid when other T cells are present, resulting in IL-2 secretion and the production of Treg cells. Peripheral Treg cells play an important role in tolerance, tumor immunity, and microbial defense. The suppressive effects of these cells are mediated via production of immunosuppressive cytokines, IL-10 and TGF-β. However, some peripherally induced Treg cells which express FOXP3 also act by cell–cell interaction. Treg cells suppress immune response during infection to avoid tissue damage but this may prolong the infection. Tumor-infiltrating CD4$^+$CD25$^+$ Treg cells are suppressive in nature and are found in increased numbers in human cancers.

Generation of peripheral Treg cells may result in the establishment of homeostasis after its disruption. The examples include infection, autoimmune diseases, certain forms of cancers, and immunodeficiency syndrome. The mechanisms by which peripheral Treg cells induce self-tolerance and homeostasis may involve cytokines or cell–cell interaction. The cytokines IL-10 and TGF-β and molecules such as CTLA-4 are involved in the effector mechanisms of Treg cells. The indirect effects of Treg cells may be mediated via antigen-presenting cells or natural killer cells. More specifically, the assembly of immunologic synapse between antigen-presenting cells and effector cells is modulated by Treg cells, which may be mediated via direct or indirect mechanisms.

The bone marrow is also a significant reservoir for Treg cells. Treg cells enter in the bone marrow and are retained through CSCR4/CSCL12 signals. Functional stromal-derived factor (CXCL12) is strongly expressed in the bone marrow and is the ligand for CXCR4. The human bone marrow CXCL12 expression is suppressed by G-CSF, and this causes the migration of Treg cells from the bone marrow to the peripheral blood. This also explains improvement in autoimmune diseases and graft-versus-host response after treatment with G-CSF. Figure 10.2 depicts the activation and differentiation of Treg cells.

10.2.3 Tr1 Cells

CD4$^+$ type 1 regulatory T (Tr1) cells were initially characterized after isolation of the peripheral blood lymphocytes from patients who suffered from combined immunodeficiency and have received successful HLA-mismatched bone marrow transplant, resulting in their generation from naïve CD4$^+$ cells following the development of antigen-specific immune response. Tr1 cells can exist naturally or may be induced. These cells are induced in the periphery when naïve T cells are



Regulates	Skin and lung inflammation	Gut homeostasis	Germinal centres	Adipose metabolism
Transcription factors	FOXP3 T-bet	FOXP3 STAT3	FOXP3 BCL-6	FOXP3 PPARγ
Chemokine receptors	CXCR3 CCR4	CCR6	CXCR5	?
Homeostatic mediators	CD40L IFNγ IL-27 IL-7	SCFA IL-10 IL-6 IL-1	?	Lipids LCFA

Fig. 10.2 Activation of Treg cells and differentiation. Treg cells expressing FOXP3⁺ migrate from the thymus and enter the secondary lymphoid tissues where they recirculate as central Treg cells. They differentiate into effector Treg cells after they are induced by TCR ligation along with costimulation with CD28 and proliferation resulting from exposure to IL-2. This effect is mediated via induction of expression of interferon regulatory factor 4. The differentiation of Treg cells also is dependent on activation of B-lymphocyte-I-induced maturation protein 1 and suppression of BTB and CNC homolog 2. There is also induction of transcription factors which along with FOXP3 induce chemokine and homing receptors, causing the polarization of Treg cells. These events are responsible for the recruitment of Treg cells to tissues or site of injury/inflammation (Source: Reproduced with permission. Liston A, Gray HD (2014) Homeostatic control of regulatory T cell diversity. *Reviews Immunology*. 14: 154–165. Nature Publishing Group)

exposed to an antigen in the presence of IL-10. Tr1 cells are generated from naïve $CD4^+$ T cells after they are activated by TCR and CD28. IL-10-producing Tr1 cells are also produced by treatment with a combination of anti-IL-4 and anti-IL-12 antibodies, dexamethasone, and active vitamin D3 (Table 10.3). Furthermore, IL-10-secreting Tr1 cells are generated when naïve $CD4^+$ T cells are treated with immature dendritic cells, IFN-α, or immunosuppressive agents. Tr1 cells are not

Table 10.3 Inducers for the generation of Tr1 cells

TCR
CD28
α IL-4 + α IL-12 antibodies
Dexamethasone
Active vitamin D3
Immature dendritic cells
IL-10
IFN-α

identified on the basis of any particular surface marker and also do not constitutively express FoxP3; however, they may express markers associated with TH2 cells and repressor of GATA (ROG). They do express high levels of surface CD152 as is the case with natural Treg cells. Tr1 cells could be either $CD4^+$ or $CD8^+$; proliferate poorly and migrate to the inflamed tissue; and secrete high amounts of IL-10, TGF-β, and IL-5 and low concentrations of IL-2 and IFN-γ; but do not secrete IL-4. In response to signaling through TCR, IL-2R, and CD28, they exhibit anergy and suppress antigen-specific proliferation of naïve $CD4^+$ cells. The immune suppression is mediated via cytokines and not cell–cell interaction. In animal models, Tr1 cells regulate mucosal tolerance, diabetes, responses to transplant antigens, infectious agents, and allergens, after they migrate to the inflamed or injured site. Their role is pivotal in the maintenance of peripheral tolerance. A member of the TNF superfamily, OX40 ligand, inhibits the generation of Tr1 cells, and as a consequence, OX40L augments immunity and abrogates tolerance.

10.2.4 ILT3⁺ Regulatory T-Cell Subpopulation

The Treg cells protect the mucosal surfaces in the lung from the allergens as they get activated in response to their exposure. The defect in the regulatory mechanism results in the development of the immune response against the allergen and subsequently the development of the atopic state, including allergic asthma. The Notch ligand, Jagged1, expressed on DCs, is responsible for TH2 differentiation by enhancing the expression of GATA-3 and IL-4 in T cells in vivo. Furthermore, the stimulus for TH2 cell-derived immune responses depends on the expression of the transcription factor IRF4 in DCs. This regulates the maturation of a PDL2-expressing DC subset 23. IRF4 also induces the expression of IL-33 and IL-10 by DCs, which is essential for the induction of TH2 differentiation. The number of PD-L2+IRF4+ DCs is substantially greater in *Csnk2b*fl/fl*Foxp3*-Cre mice than in *Csnk2b*fl/fl mice, implying that CK2β controls the ability of Treg cells to regulate PD-L2+IRF4+ DCs and TH2 immune responses.

A Treg cell subpopulation identified by the presence of immunoglobulin-like transcript 3 (ILT3) provides inhibition of the main function of Treg cells. Thus the activity of this subpopulation of Treg cells can be regulated. Protein kinase CK2 is involved in the suppression of excessive TH2 response in the lung upon exposure to allergen by Treg cells. CK2 is a highly conserved serine–threonine kinase and takes part in many signal transduction pathways, including the NF-κB, PI (3)K, and Wnt pathways. By using Treg cell-specific gene targeting, it is observed that the inhibition of allergic immune response by TH2 cells is protein kinase CK2 dependent. There is proliferation of ILT3+ Treg cells as a result of genetic ablation of the β-subunit of CK2 in Treg cells. ILT3+ Treg cells subpopulation does not inhibit the development of IRF4+PD-L2+ dendritic cells. These dendritic cells play a major role in the development of TH2-mediated immune responses. A different explanation for the TH1/TH2 imbalance favoring TH2 cells is that during inflammation CK2β deficiency results in the reprogramming of Treg cells into TH2 cells.

ILT3 has been identified as a CK2-controlled protein that regulates Treg cells and modulates TH2 cell-induced inflammation. Negative signals are transduced by ILT3, which impair TCR-induced pathway. This causes activation of tyrosine phosphatases (SHP-1 and SHP-2) and as a result dephosphorylating of Zap70. The TCR signaling is much lower in $ILT3^+$ Treg cells than $ILT3^-$ Treg cells. The expression of all three receptors of the Nr4a family is lower in $ILT3^+$ Treg cells than $ILT3^-$ Treg cells. Deletion of Nr4a family receptors results in defective thymic Treg cell development and in a TH2 cell-induced inflammation, which is fatal in animal models. In contrast, the attenuation of suppression or deletion of SHP-1 increases the inhibitory function of Treg cells. The polymorphisms in the *LILRB4* locus are associated with high IgE levels in the serum of children with asthma. There appears to be a close association between ILT3-dependent signaling and Treg cell-induced suppression of inflammatory responses caused by TH2 cells. CK2β deficiency leads to increased expression of ILT3 and inhibition of the activity of Treg cells; this results in the proliferation the TH2 cell-inducing IRF4+PD-L2+ DC population. As a result there is an induction of TH2 cells that leads to allergy.

10.2.5 TH3 Cells

Antigen-specific Treg cells, TH3, are generated from naïve $CD4^+$ T cells as a result of low doses of antigen via the oral route. This phenomenon is just opposite to hyporesponsiveness resulting from anergy or deletion, resulting following exposure to high antigen doses. Oral tolerance results from such exposure as a result of interaction of dietary antigens with GI immune apparatus. TH3 can also be induced in periphery. They are TGF-β-producing cells, which may also express FOXP3. TH3 cells suppress antigen-specific responses and can transfer tolerance. Their mechanism of action is mediated via TGF-β, and defects in TH3 cells may be associated with the development of autoimmune disease.

10.2.6 n Regulatory T Cells (nTreg) and i Regulatory T Cells (iTreg)

nTreg are derived in the thymus from Foxp3^{-}CD25^{+}CD4^{+} cells. iTreg develops outside the thymus from FOXP3^{+} CD25^{-} CD4^{-} cells, in chronically inflamed tissues such as spleen, lymph node, and gut-associated lymphoid tissue, from naïve T cells. Both cell types become FOXP3^{+} CD25^{+} CD4^{+}. The activation of nTreg requires CD28 for costimulation, and the activation of iTreg requires CTLA-4 for costimulation. Both are inhibitors of the autoreactive T-cell signaling and cell–cell interaction dependent as well as independent signaling.

10.2.7 Natural Killer T (NKT) Cells

Since NKT expresses a TCR, they are defined as T lymphocytes and are distinct from NK cells, although they both share CD161 or NKR-P1. They also differ from T lymphocytes and other Treg cells because they lack the ability to interact with peptide antigens in classical MHC Class I or Class II restricted manner. Instead, they recognize antigens in context of a glycolipid, which is a nonclassical antigen-presenting molecule called CD1d. NKT cells have two main subsets, CD4^{+} and CD4^{-}, but some of the NKT cells are also CD8^{+}. The subsets of NKT cells are present along with other lymphocytes, but their numbers are tissue dependent. In mice, their numbers are most prevalent in the liver and with lower frequencies in the bone marrow, spleen, thymus, blood, and lymph nodes.

They recognize a class of antigens that is not recognized by T lymphocytes. After recognition of the antigen, they are activated within 1–2 h of TCR ligation and produce both TH1 (IFN-γ, TNF-α) and TH2 (IL-4, IL-13) cytokines. Many glycolipids including glycophosphatidylinositol, gangliosides, and phosphoethanolamine can activate NKT cells. Although their cytokine production pattern is TH0-like, they can produce either a TH1 or TH2 immune response.

NKT cells express receptors both for the NK lineage and for T cells (TCR $\alpha\beta$) and thus are a unique population of lymphocytes. Tumor cells expressing lipid antigens are recognized and killed by NKT cells. These lipid antigens related to the glycolipid α-galactosylceramide are presented to NKT cells in context of MHC (Ib, CD1d) molecules. In addition to their ability to kill tumor cells, they also regulate autoimmune diseases. Their effector function as natural killer cells and the ability to secrete a number of cytokines including INF-α, TGF β, IL-4, and IL-10 are enhanced following antigen recognition by the TCR in context of MHC Class Ib molecules. On the basis of secretion of cytokines, it appears that NKT cells may be involved in modulating both innate- and TH2-dependent acquired immune response. In animal models, NKT cells have been shown to inhibit the onset of type 1 diabetes mellitus and multiple sclerosis, and their depletion accelerates the development of the disease. In humans, there is also an association of NKT cells and autoimmune disease.

A decrease in the frequency of Vα24-J αQNKT cells is associated with relapse in patients with multiple sclerosis, and the patients with diabetes also have lower expression of Vα24-JαQ NKT cells.

NKT cells also play a role in allograft tolerance, which involves NKT cell-dependent allospecific regulatory T-cell generation. They induce cardiac allograft tolerance and inhibit graft-versus-host disease. NKT cells may also play a role in tumor rejection and are also required for IL-12-mediated cancer therapy. A-GalCer, a glycolipid recognized by NKT cells, causes rejection of a number of tumor cell lines via activating NKT cells.

NKT cells are unique regulatory cells although they also act as effector cells. Their regulatory role is reflected by the pattern of cytokines they secrete, interaction with dendritic cells, and their small numbers. Interaction with dendritic cells suggests their role in acquired immune response. It seems that the phenotype of these cells may be distinct at different locations such as the thymus versus the liver, which may be a deciding factor in their ability to promote or suppress the immune response. The human $CD4^+$ NKT cells produce a high TH2/TH1 cytokine ratio with distinct expression of cytotoxic and chemokine receptors as opposed to $CD4^-$ NKT cells. The type of signal that NKT cells receive may also determine whether they will produce pro- or anti-inflammatory response. The examples include the production of INF-γ when cross-linked with IL-12 or anti-NK1.1 and IL-4 production after cross-linking with IL-7; therefore, the cytokines secreted by NKT cells may be dependent on the type of TCR stimulation. The OCH analog of A-GalCer induces TH2 responses and C-glycoside induces TH1 responses. Alternatively, the products produced by NKT cells may be the same, but different physiologic or pathologic circumstances may interpret them differently and thus NKT cells are not responsible for the final impact. The example includes the immediate production of both INF-γ and IL-4 following a-GalCer stimulation, which are the products of preformed mRNA, but IL-4 secretion stops within a few hours while NKT cells continue to secrete IFN-γ for another 2–3 days. This suggests that this differential response may be attributed to the temporal nature of the interactions of NKT cells with other immune cells.

$CD4^+CD25^+$ T cells and NKT cells exist as natural suppressor cells from the early fetal life before antigen exposure and play an important role in immune regulation during innate and/or primary immune responses. The NKT cells recognize glycoproteins via Vα chain of TCRαβ, which is expressed by tumor cells, pathogens, injured apoptotic cells, and blast cells. The primary immune response balance of TH1 and TH2 cells is modulated by NKT cells via their secretion of IL-4 and IL-10. Naturally occurring $CD4^+CD25^+$ T-cell subset is also present in the peripheral lymphoid system and without antigen stimulation can affect the primary immune response. Their effect is mediated via cell–cell interaction as well as the secretion of TGF-β. Antigen recognition activates NKT cells, which respond by secreting IL-13. IL-13 receptors are expressed on certain myeloid cells, and these cells, as a result of IL-13 binding and signal transduction, produce TGF-β and suppress the $CD8^+$ CTLs. This results in the suppression of tumor immunity since $CD8^+$ CTLs kill tumor cells.

10.2.8 Regulatory CD8⁺ T Cells

Most of the information about Treg cells involve $CD4^+$ sublineage with regulatory activity. Following the identification of $CD4^+$ Treg cells, a subpopulation of $CD8^+$ T cells was identified, which suppressed helper T cell and B cell responses in an MHC-dependent manner, requiring the expression of HLA Class Ib MHC molecule Qa-1 on target cells. $CD8^+$ Treg cells in mice are divided at least into two groups: Qa-1 restricted and Qa-1 nonrestricted; Qa-1 is an equivalent of human HLA-E.

$CD8^+$ Treg cells are activated by autologous $CD4^+$ T cells after their induction during the primary immune response, differentiating into functional suppressor T cells. Their effector function is prominent during the secondary immune response as well as memory-based immune responses. $CD8^+$ Treg cells may be responsible for producing suppression to autoimmunity after the patient recovers from the first episode of the disease and consequently will resist to a relapse and may decrease the severity of the symptoms in future episodes of the same disease. Qa-1-restricted $CD8^+$ Treg cells recognize their target through their TCRαβ in an MHC-restricted manner. Some Qa-1 self-peptide-expressing activated T cells are downregulated by these cells, but this is not the case for all activated T cells. Both types of Qa-1 receptors, TCR and CD94/NKG2, can be expressed on $CD8^+$ Treg cells. CD94/NKG2 is a C-type lectin receptor present on NK and $CD8^+$ cells. The TCR can recognize Qa-1 complex on induced $CD4^+$ cells, resulting in suppressor activity, and CD94/NKG2 recognize Qa-1/Qdm ligands. NKG2 receptors play a dual role as they can either enhance suppression in response to NKG2C, E, or H or inhibit suppression in response to NKG2A or NKG2B. A non-Qa-1-restricted $CD8^+CD28^-$ Treg cell subset has been identified, which mediate suppression via antigen-presenting cells.

Human $CD8^+$ Treg cells express CD25, CD69, CTLA-4, and FOXP3. They secrete IL-4, IL-5, IL-13, and TGF-β but do not secrete IFN-γ and contribute to immunoregulation. Naïve $CD8^+CD25^-$ cells are considered to differentiate into $CD8^+$ Treg cells when presented with an antigen. $CD8^+CD28^-$ Treg cells are induced in the presence of IL-10. IL-10 may be involved in the downregulation of dendritic cell costimulation as well as in the upregulation of ILT-3 (immunoglobulin-like transcript 3) and ILT-4. An additional human Treg subset has been identified, which includes $CD8^+$, $LAG\text{-}3^+$ (lymphocyte activation gene 3, an MHC class II-binding CD4 homolog), $CD25^+$, $FOXP3^+$, $CCL4^+$, and it suppresses T-cell responses via secretion of chemokine CC chemokine ligand 4.

$CD8^+$ Treg cells are generated in neonatal life when T lymphocytes enter into nonlymphoid tissue and maintain tolerance during adulthood. The thymus does not contribute to this antigen-specific tolerance, and the continued presence of the antigen is necessary to maintain tolerance. Antibody-mediated inhibition of T-cell migration abrogates this tolerance. TGF-β1 may play a role in the upregulation of this TCR^+CD8^+ subset-mediated tolerance. Furthermore, granzyme B is activated in this TCR^+CD8^+ Treg cell subset, which has been implicated in the induction of cell

death of effector T cells by $CD4^+CD25^+$ Treg cells. This naturally occurring TCR^+CD8^+ Treg cell subset is induced by self-antigens that are expressed in neonatal mice on parenchymal cells. They maintain tolerance during adult life as a result of the downregulating effector function of T cells. This mechanism is independent of $CD4^+$ T cells.

10.3 Regulatory T Cells in the Mucosal System

Tolerance is an important goal of the immune response in the gastrointestinal tract. Harmful pathogens are recognized by the mucosa-associated lymphoid tissue to protect the epithelial layer from their deleterious effects. Moreover, the mucosa-associated lymphoid tissue develops tolerance against dietary and bacterial antigens. In normal individuals, a number of regulatory cell types control inflammatory response when pathogenic bacteria and viruses attack the intestinal mucosa. A lack of appropriate regulatory responses, which limit inflammation in the gut results in the development of inflammatory bowel disease. A number of Treg cell subsets may be involved in mucosal immunity including CD45Rbl0, $CD4^+CD25^+$, $CD4^+$, $CD103^+$, $CD4^+$Tr1, $CD4^+CD8^+$IELS, TCRαβ$^+$ $CD8^+$IELS, and $CD8^+CD28^-$ cells. $CD4^+$Tr1 are present in intestinal mucosa, and their immunosuppressive effects are mediated via IL-10, which they produce in large amounts. They are induced by IL-10, secreted by intestinal epithelial cells and other Treg cells in intestinal mucosa. The generation of mucosal $CD4^+$ Tr1 cells is negatively modulated by a subset of dendritic cells. The role of these cells in the prevention of human inflammatory bowel disease has not yet been established. Another subset of Treg cells associated with mucosal immunity is $CD4^+$TH3. These cells are present in the human intestinal mucosa and play a role in controlling inflammation in the gut.

The intestinal epithelial cells play an important role in the generation of these Treg cells that maintain tolerance in the mucosal immune system because of their ability to serve as antigen-presenting cells. The intestinal epithelial cells process and present antigenic fragments in context of the MHC molecules to the TCR. Several regulatory subsets, which include both $CD4^+$ and $CD8^+$ T cells, are involved in oral tolerance, and most of the immune suppression, which they cause is mediated via IL-4, IL-10, and TGF-β. The mechanism of tolerance resulting from antigen exposure in the lamina propria is different from oral tolerance. This tolerance is not dependent on perforin, and these cells act like $CD4^+$, $CD25^+$, or $CD8^+$ and $CD28^-$ Treg cells and express CD8, CD101, and CD103. These $CD8^+$ Treg cells are not present in patients with inflammatory bowel disease, which may be due to the epithelial cell glycoprotein gp180, a molecule expressed on all normal intestinal epithelial cells. The interaction of the gp180/CD1d complex on intestinal epithelial cells with a subset of $CD8^+$ Treg cells results in oligoclonal expansion of $CD8^+$ Treg cells in the intestinal mucosa.

10.4 T-Cell Vaccination and Regulatory T Cells

Anti-idiotypic and anti-ergotypic Treg cells are activated after T-cell vaccination and are regulators of the immune response. The regulatory T cells induced by activated T-cell vaccines, which are not anti-idiotypic, are called anti-ergotypic. They proliferate in response to autologous T cells after their activation, and their presence does not require T-cell vaccination or prevalence of an autoimmune disease. They are widely distributed in the thymus, spleen, and lymph nodes in naïve rats, and they do not need antigen exposure. Anti-erg T cells include both TCR α/β^+ and TCRγ/δ^+ T cells, and $CD8^+$ markers are present on naive anti-erg T cells. IFN-γ and TNF-α are secreted by TCRγ/δ^+ anti-erg T cells following activation of T cells. No detectable levels of cytokines are produced by TCR α/β^+ anti-erg T cells as they proliferate in response to T-cell activation. Cell–cell interaction is involved for interaction between anti-erg T cells and activated stimulator T cells. Anti-erg T cells can also recognize ergotype on the cell surface of macrophages and other antigen-presenting cells, but this results in a much weaker response as opposed to the activated stimulator T cells. The naïve TCR α/β^+ $CD8^+$ and TCRγ/δ^+ anti-ergotypic T cells respond in a classical MHC Class I restricted manner, and the B7 and CD28 molecules are involved in this recognition process. $CD4^+CD25^+$ Treg cells do not play a role in anti-ergotypic response.

A regulatory cell needs to meet two conditions in order for it to be called an ergotype. It must be expressed and presented by the activated and not the resting cells, resulting in the activation of anti-erg T cells; TCR, CD25, and HSP60 epitopes are some examples of ergotopes. Only activated T cells can present ergotypic TCR peptides to anti-erg T cells, despite the expression of TCR on resting T cells.

Anti-id Treg cells utilize unique TCR CDR3 peptides on the cell surface of effector cells. Anti-idiotypic T cells generated after T-cell vaccination are cytolytic T cells, which are $CD8^+$ that kill after interaction with target T-cell receptors in context with MHC Class I molecules. This $CD8^+$ anti-idiotypic T-cell response is responsible for the depletion of circulating autoreactive T cells. Furthermore, $CD4^+$ regulatory T-cell responses also occur in response to T-cell vaccination in addition to the generation of $CD8^+$ anti-idiotypic T cells. The production of $CD4^+$ Treg cells in anti-idiotypic response may be responsible for the production of T-cell vaccination-induced clinical effects. The precise mechanisms relating to the involvement of $CD4^+$ Treg cells in these processes have not yet been established. However, interaction with ergotypes including IL-2 receptors and heat shock protein 60 may result in the induction of $CD4^+$ Treg cell responses.

In clinical trials of multiple sclerosis, the therapeutic effects of T-cell vaccination involve $CD4^+$ Treg cells, which are produced as a result of repeated immunization with irradiated autologous T cells selected for autoantigens. The MS patients receiving T-cell vaccination produce two different populations of Treg cells that differ in their expression of FOXP3 gene and cytokine production and may have different mechanisms of action. Most of the cells have an abundant expression of FOXP3 gene and produce IL-10 and INF-γ, while a small number of cells produce only

IL-10 and have very low levels of FOXP3 gene. After T-cell vaccination, Treg cells expressing CD4CD25FOXP3 may be derived from the naturally occurring $CD4^{+}CD25^{+}$ Treg cells. The T-cell vaccination results in the upregulation and proliferation of $CD4^{+}$ $CD25^{+}$ Treg cells, the numbers of which are below normal in patients with MS. The other subset of Treg cells, $CD4^{+}$ $CD25^{+}FOXP3^{-}$, produces high levels of IL-10, resulting in the suppression of activated T cells. This inhibition by IL-10 is reversed by IL-10 antagonist or monoclonal antibody to IL-10. The $CD4^{+}$ $CD25^{+}FOXP3^{+}$ Treg cells recognize an epitope corresponding to 61–73 residues of α chain of IL-2 receptors. This may be clinically relevant in finding new treatments for autoimmune diseases.

10.5 Regulatory T Cells and Antibody Production

The autoantibody production in autoimmune diseases may be attributed to the inability of Treg cells to control their synthesis. In an autoimmune model, T cells regulate the mechanisms through which B cells that were autoreactive to self-antigens do not produce autoantibodies, suggesting a role for suppressor T cells. The administration of irradiation, thymocytes, lymph nodes, or spleen cells inhibits the production of autoantibodies, which is attributed to the suppressor T cells.

10.6 Mechanisms of Induction of Treg Cells

Treg cells are induced by low doses of oral antigen, but high antigen doses result in anergy. $CD4^{+}$ and $CD8^{+}$ Treg cells can be produced in autoimmune murine models when self-antigen is administered by the oral route. These antigen-specific Treg cells are of the TH_3 subgroup. Antigen-specific Treg cells can also be induced when nonobese diabetic mice are administered human insulin orally or by aerosol. This causes these animals to be hyporesponsive to human insulin when an immunostimulatory route is used. $CD8^{+}$ Treg cells are produced in kidney grafts in rats after oral exposure to alloantigen, and transfer of these cells to naïve animals will prolong graft survival. Allogeneic cardiac graft survival is prolonged following intratracheal delivery of allogeneic peptides. This is mediated via production of IL-4- and IL-10-secreting Treg cells.

The antigen recognition by TCR involves activation of multiple signal pathways involving additional ligands. CD40 ligand (CD154), expressed on $CD4^{+}$ T cells, is important in initiating costimulatory signals after it binds to CD40 on antigen-presenting cells. After activation of CD40, other molecules including CD80 and CD86 are upregulated, resulting in the proliferation of T cells and generation of an immune response. CD80 and CD86 also serve as receptors for TCRs, CD28, and CTLA-4. CD28 is an activator of the immune response via IL-2 secretion and induction of T-cell proliferation, whereas CTLA-4 inhibits T-cell responses. Consequently,

CTLA-4 is involved in inducing immune tolerance by inhibiting the signals which are responsible for T-cell activation in response to an antigen and result in the induction of Treg cells.

Alloantigen-specific Treg cells can also be generated by a number of other ligands. Administration of anti-CD40LmAb to antagonize CD40-CD40L pathway results in the generation of alloreactive T-cell responses by cell–cell interaction. The antagonism of downstream signal transduction such as inhibition of nuclear factor κB results in the generation of Treg cells. Agonist-like signals can also be used to generate Treg cells. For example, LFA-3- or CD58- mediated engagement of CD2 on naïve $CD4^+$ T cells results in the differentiation of Treg cells that are HLA specific. Similarly, the induction of inducible costimulatory molecule on T cells and Notch during antigen presentation will result in the generation of Treg cells. But the most potent positive signals for their generation are provided by cytokines, specifically IL-10 and TGF-β. IL-10 downregulates expression of CD40, CD80, and CD86, resulting in inhibition of generation of $CD4^+$ and $CD8^+$ cells. This environment is optimal for the induction of Treg cells. Other proteins and soluble peptides could also produce antigen-specific Treg cells.

10.7 Antigen Specificity of Regulatory T Cells and Mechanisms of Suppression

Antigen-specific Treg cells function through antigen presentation, activation, and recognition of target cells. The antigen presentation is achieved in context of MHC molecules and in association with costimulatory and regulatory signals. After induction, coming in contact with the same antigen renders functional activity in Treg cells, which is followed by antigen-specific recognition of target cells by Treg cells.

Treg cell-induced suppression is mediated by several different mechanisms. The downregulation of CD40, CD80, and CD86 molecules results in a lack of T-cell activity when inhibited by $CD8^+CD28^-$ Treg cells. Another mechanism is mediated via cytokines, IL-10 and TGF-β, secreted by antigen-activated Treg cells. IL-10 downregulates CD80 and CD86 molecules via activation of JAK/STAT pathways and inhibits NFκB activation. As a result, T-cell activation and IL-12 production are affected. The effects of TGF-β are mediated via Smad complex. Another proposed mechanism includes the killing of the effector $CD8^+$ T cells, which kills the graft by Treg cells that involves Fas–Fas ligand pathway.

10.8 FOXP3 Expression and Regulatory T-Cell Activity

The function of Treg cells is controlled by FOXP3 gene, an X-chromosome-linked factor, in a binary function, resulting in the maintenance of immune tolerance. The immune-suppressive activities of T cells are regulated by FOXP3 gene. As a

consequence, most attention has focused on equating abnormalities in Treg cells with immunologic diseases. The effector function of Treg cells is as important as their numbers in regulating the immune response. For example, in diabetic NOD mice, there are lower levels of FOXP3 in Treg cells of intra-islet as opposed to other peripheral lymphoid organs, but the frequency of Treg cells expressing this gene in different parts of the body is not different. The differences in the level of expression in diabetic NOD mice are not found in other mice strains, which are not susceptible to diabetes. One of the regulatory mechanisms may be the degree of gene switching, which determines expression levels of FOXP3. Consequently, immune disease may be a result of decreased FoxP-3 expression. It seems that decreased FOXP3 expression causes defects in the function of Treg cells, and their differentiation into effector cells results in an augmented immune response that produces a loss of tolerance and probable development of the autoimmune disease.

10.9 Toll-Like Receptors (TLR) and Regulatory T Cells

Innate and acquired immune response are induced and regulated by the TLR. MyD88, a protein associated with TLR-mediated signal transduction pathway in dendritic cells, is involved in the suppression of Treg cell activity, resulting in an augmented immune response. TLR signaling is required for the maturation of dendritic cells, and mature dendritic cells are potent inhibitors of Treg cell function. However, mature dendritic cells induce Treg cells expansion in association with TLR, IL-1, and IL-6. Small doses of IL-2 are required to maintain the suppressive function of Treg cells, which is inhibited by high doses of IL-2. The mechanism of IL-2-mediated loss of Treg cell activity is not known and is not mediated via FoxP-3 gene. Furthermore, IL-6 and the strength of TCR signal help overcome the suppressive effects of Treg cells on effector cells. The suppression of Treg cells by TLR is attributed to TLR-2, which can recognize bacterial lipoproteins, and the removal of the TLR-2 influence results in the reestablishment of the suppressive abilities of Treg cells. TLR-2 is expressed on $CD4^+$ and $CD8^+$ T cells and can activate TCR-primed T cells as well as memory T cells. Treg cells and effector T cells are distinctly regulated by TLR-2-dependent signal transduction. Although T-cell function is not affected by TLR-2 signaling alone, the proliferation of TCR-primed Treg cells is strongly augmented by the agonists of TLR-2 which makes the Treg cells temporarily inactive. During infection bacterial lipoproteins also increase the proliferation and IL-2 production from the TCR-triggered effector cells. This IL-2 increases the proliferation of both the effector and Treg cells; however, Treg cells are not able to suppress the effector cell function, which is a unique mechanism by which TLR regulates the function of Treg cells. After the bacterial infection is under control and the pathogens have been eliminated, Treg cells regain their suppressive function and IL-6 plays a role in this process. Consequently, this avoids the development of autoimmune disease that may result from the unregulated activity of effector T cells.

10.10 CTLA-4 and Regulatory T Cells

CTLA-4 is constitutively expressed on Treg cells; its deficiency is associated with fatal autoimmune proliferative disease, and inhibition of CTLA-4 by specific antibodies results in the development of autoimmune disease. Its polymorphism has a role in the development of autoimmune diseases including diabetes, Addison's disease, and thyroid disease. Treg cells are involved in the development of disease resulting from antagonisms of CTLA-4, which may be the result of depletion of Treg cells by antibodies to CTLA-4. The activation of $CD4^{+}CD25^{-}$ T cells as a result of blockade of CTLA-4 receptors may block the suppressive effects of Treg cells on $CD4^{+}CD25^{-}$ T cells.

10.11 T-bet, GATA-3, and Regulatory T Cells

The function of the expression of T-bet and GATA-3 on regulatory T cells is of interest. Expression of T-bet and GATA-3 is regulated by the environment of cytokines. The deletion of genes for either T-bet or GATA-3 does not affect Treg function. However, when both are deleted, there is development of severe autoimmune-like disease in animal models. There is reduced expression of FOXP3 and increased expression of RORϒt, which correlates with the loss of Treg function. In the steady state, Treg cells transiently upregulate one of the two transcription factors for maintaining T-cell homeostasis.

10.12 Regulatory T Cells and Disease States

10.12.1 Allergic Disease

The role of Treg cells in the prevention of allergic disease and asthma is of considerable interest as the prevalence of these diseases continues to rise. On the basis of the ability of Treg cells to prevent sensitization to allergens, they could be potentially used for the treatment of the allergic disease, and the prevention and regulation of TH2-mediated responses may be possible. Mouse $CD4^{+}CD25^{+}$ T cells, after preactivation with differentiated TH2 cells, inhibit TH2 cytokine production and suppress TH2 cell differentiation from naïve $CD4^{+}$ T cells without the requirement of cytokines. $CD25^{+}$ cells also inhibit IgE production in transgenic mice with monoclonal populations of T and B cells, and Tr1 cells inhibit TH2 sensitization and IgE production provided that their adoptive transfer is before sensitization.

In humans, undesired TH2 responses to environmental allergens are prevented by Treg cells, and the allergic disease may result from inadequate suppression of unwanted TH2 responses both by naturally occurring Treg cells and Tr1 cells.

An overall defect in regulatory ability of Treg cells is not present in atopic individuals but a diminished suppressive ability of $CD4^{+}CD25^{+}$ T cells is observed in atopic individuals when compared with their nonatopic counterparts. Treg cells from both the asthmatic and non-asthmatic individuals have the same ability to suppress anti-$CD3^{-}$- and anti-$CD28^{-}$-stimulated cytokine production. Nonetheless, T-cell activation by allergens is suppressed by Treg cells, and atopic individuals may be deficient in these regulatory mechanisms. Nonatopic individuals possess higher numbers of allergen-specific IL-10-producing $CD4^{+}$ cells as compared to the atopic individuals. In addition, allergen-activated TH2 cells can be inhibited by IL-10-producing T cells, and this can be reversed by either anti-IL-10 or TGF-β.

The mechanisms that alter the intricate balance between regulatory and suppressive responses after allergen exposure have not been elucidated, although a number of possibilities exist that may lead to atopic state. According to the hygiene hypothesis, microorganisms alter antigen-presenting cells, which may result in the production of Treg cells when exposed to allergens. As a consequence, it is feasible that regulation by Treg cells is restricted by LPS-induced activation of Toll-like receptors, specifically TLR4. IL-10-producing Treg cells are also involved in the suppression of allergic responses as a result of prior exposure to allergens and mycobacterial antigens. In young children, exposure to cat antigens protect them from later development of allergies to cats due to a dominant IL-10 response resulting in modified TH2 responses where it seems that both Treg and Tr1 cells are responsible in developing tolerance.

Corticosteroids remain the main hallmark for the treatment of allergic disease/asthma despite their adverse side effects. While their administration via inhalers and nebulizers has alleviated some concerns because of their less detrimental side effects, the development of drugs that will induce Treg cells will provide an attractive alternative. Corticosteroids, in addition to inhibiting TH2 cytokines, stimulate Tr1 cells and increase the effector function of Treg cells by increasing IL-10 production. Inhaled corticosteroids also increase Foxp3 expression in asthmatics. Glucocorticoid-resistant asthma patients have impaired Tr1 cells, and they do not show improvement after treatment with corticosteroids. These observations provide a rationale for the development of a new class of drugs that may selectively increase the effector function of naturally occurring as well as IL-10-induced Treg cells and enhance FoxP-3 expression. The concept is further strengthened by the observation that immunotherapy for allergic disease results in induction of IL-10 production from Treg cells, and these patients have increased numbers of IL-10-producing Treg cells after they have received injections for allergen extracts.

Lastly, under naturally induced circumstances of tolerance such as beekeepers receiving multiple bee stings or children who are no longer allergic to cow's milk, there is an increase in IL-10-producing Treg cells. The evidence is convincing that Treg and Tr1 cells regulate responses to allergens in nonatopic individuals and their function may be impaired in atopy, specifically after prolonged antigen exposure. This suggests the need for the development of either new corticosteroid-like drugs, which only target these mechanisms or alternate novel immunotherapeutic regimens. In Fig. 10.3 is shown the association of Treg cells with various disease states.

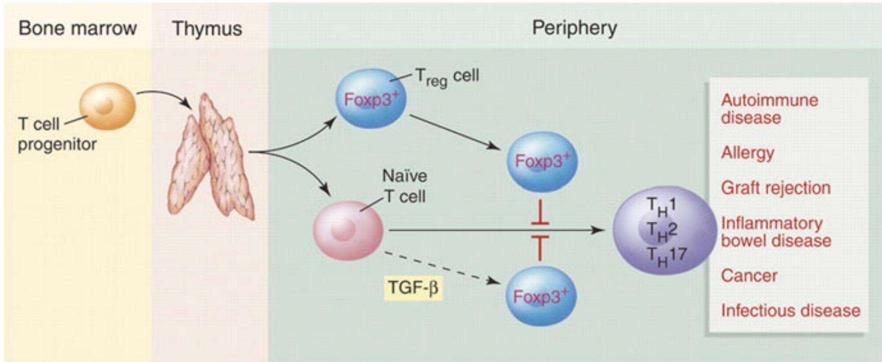


Fig. 10.3 Treg cells and disease states. This figure depicts the association of Treg cells with some disease states. The induction and proliferation of naïve T cells and their differentiation to effector T cells is inhibited by natural Treg cells (*FOXP3*⁺). This includes the production of TH1, TH2, and TH17 cells. These subsets of T cells play a role in normal biological function as well as in a number of disease states (Reproduced with permission. Source: Sakaguchi S, Powerie F (2007) Emerging challenges in regulatory T cell function and biology. *Science* 317: 627–629. American Association for the Advancement of Science)

10.12.2 Autoimmune Diseases

Human autoimmune diseases are a number of complex genetic disorders strongly associated with the MHC complex on the chromosomes. Defects in the function of $CD4^+CD25^+$ Treg cells are associated with various autoimmune diseases including multiple sclerosis, type 1 diabetes, and myasthenia gravis. The patients with multiple sclerosis have a significant decrease in the effector function of Treg cells, despite no differences in their frequency as compared to the normal controls. The patients with rheumatoid arthritis and juvenile idiopathic arthritis also have altered $CD4^+CD25^+$ Treg cells. Adult patients with rheumatoid arthritis exhibit high numbers of Treg cells in synovial fluid as opposed to the peripheral blood. The patients with juvenile arthritis disease have similar increases of Treg cells in the synovial fluid. These cells also contain CD27 marker with higher expression of Foxp3. Treg cells function differentially in various autoimmune diseases; for example, in human autoimmune polyglandular syndromes and multiple sclerosis, the function of Treg cells is decreased, whereas Treg cells isolated from patients with autoimmune arthritic diseases exhibit augmented effector function.

10.12.3 Inflammatory Diseases

The expression of Id2 and Id3 in Treg cell is responsible for the inhibition of the development of fatal inflammatory disease. It has been reported that TCR-induced signaling at first inhibited Id3, causing the stimulation of follicular regulatory

T-cell-dependent transcription signature. But continued lower levels of Id2 and Id3 infringed with follicular regulatory T-cell development. The maintenance and localization of the Treg cell population was inhibited by the suppression of id2 and id3 expression. This suggests that Id2 and Id3 regulate follicular regulatory T-cell checkpoints and modulate the survival and homing of Treg cells.

The ability of Treg cells to suppress the function of various effector T cells has not been clearly established. A PTEN-mTORC2 axis is responsible for the maintenance of Treg cells and modulates the regulation of effector cells by Treg cells. The phosphatase PTEN is involved in the stability of Treg cells and suppression of the activity of TH1 cells and follicular helper T cells. When PTEN is removed from the Treg cells, there is increased follicular helper T-cell response, resulting in spontaneous inflammatory disease. This is resolved by inhibition (deletion) of IFNϒ, suggesting that the control of TH1 cells and follicular helper T cells is coordinated. PTEN is responsible for maintaining the metabolism and stability of Treg cells. Furthermore, the deficiency of PTEN results in the upregulation of the metabolic checkpoint kinase complex mTORC2 and Akt (serine–threonine kinase), and its inhibition will renew the effector function of PTEN-deficient Treg cells.

10.12.4 Infections

Infections present a challenge to the immune system which requires measured pro-inflammatory anti-infectious agent response without being detrimental to self. This intricate balance is subject to control by Treg cells and a role of Treg cells in chronic viral and bacterial infections has been suggested. An increase in peripheral Treg cells is observed in patients with hepatitis B and C infections. Furthermore, Treg cells prevent antiviral response, since T-cell responses to HCV, HBV, and HIV antigens and cytomegalovirus are induced after removal of Treg cells from peripheral blood of patients with viral infection (Table 10.4). Treg cells have a protective effect in HIV infection, where a decrease in Treg cells results in immune hyperactivity in HIV-affected patients. A strong HIV-specific Treg cell activity is associated with lower levels of virus in plasma and higher $CD4^+/CD8^+$ ratios in HIV-infected patients. Consequently, intact Treg cell activity is desirable in patients infected with HIV.

Following HIV infection, Treg cells control the levels of the activation of the immune response to avoid immune system exhaustion as well as tissue damage due to a robust immune response. However, this causes the dysfunction of immune response specifically due to inhibition of the generation of HIV-specific effector cells. Furthermore, the role of Treg cells changes during the different stages of HIV infection, as HIV infection causes alterations in the frequencies of Treg cells in the peripheral blood. As the disease progresses, there is a decrease in the frequency of Treg cells in the peripheral blood despite elevated $CD25^{bright}$ expression on $CD4^+$ T cells, which may be attributed to their expression of CD4 and other chemokine receptors that are targets for HIV.

Table 10.4 Treg cells and viral infections

Treg type	Impairment of antiviral response
Treg cells	HCV, HIV, HSV
Tr1 cells	EBV, HCV, MLV
CD8+ Treg cells	HCV, HIV
NKT cells	HIV

Chronic infection such as HIV results in immunosuppression as a result of induction of $CD4^+$ Treg cells. Increased risk of Kaposi's sarcoma, non-Hodgkin's lymphoma, and liver cancer is associated with long-term infection such as HIV. Immunosuppression in HIV-infected individuals occurs before the development of AIDS, which proceeds before the depletion of $CD4^+$ T cells, and the induction of Treg cells may play a role in this process. The mechanisms are IL-10 independent and include the involvement of TGF-β secreted via signaling through cell–cell interaction involving CTLA-4.

$CD8^+$ T-cell response plays an important role in the viral replication of hepatitis B virus (HBV) resulting in liver damage. These $CD8^+$ T cells are virus specific and a defect is found in these cells in the patients with chronic HBV infection as opposed to the recovered individuals. This may be due to a high antigen dose deletion and a lack of help from $CD4^+$ T cells or may be a result of Treg cells. For example, in herpes simplex virus-infected mice, the clonal expansion and effector function of virus-specific $CD8^+$ T cells is augmented by Treg cells, suggesting that Treg cells can be modulated in the periphery after viral infection. Another example is the regulation of hepatitis C virus (HCV)-specific T cells by Treg cells in patients with HCV infection. The circulating $CD4^+CD25^+$ Treg cells in patients with HBV infection suppress activation of HBV-specific $CD8^+$ T cells. This may also serve as a feedback to avoid excessive pathogenic responses in chronic HBV infection and also helps to avoid complete clearance of the viral antigen in patients who have resolved HBV infection.

10.12.5 Cancer

Immune dysfunction and poor tumor-specific immune responses are observed in cancer patients with enhanced Treg cell activity. Furthermore, many different types of tumors possess high frequency of Treg cells that inhibit a variety of immune functions including T-cell proliferation, cytokine production, and cytotoxic activity. Regulatory T cells play an important role in the escape of tumor cells. The expression of CD69 is induced on T lymphocytes and natural killer cells. CD69 is rapidly and transiently expressed on activated but not on resting lymphocytes and functions as signal-transmitting receptors in lymphocytes. It may produce an activating function to cause pro-inflammatory responses but also produces regulatory function. The number of $CD69^+CD4^+$ $CD25^-$ T cells increases dramatically during the

progression of the tumor. As opposed to other regulatory T-cell subsets, they express high numbers of CD122 but do not express FOXP3, and they secrete IL-2, IL-10, IFN-ϒ, IL-10, and TGF-β1. $CD69^{+}CD4^{+}$ $CD25^{-}$ T cells inhibit proliferation of T cells via cell–cell interaction. The antibodies directed against TGF-β1 neutralize their suppressor function. These cells express high levels of membrane-bound TGF-β1, which plays a role in the inhibition of T-cell proliferation. In addition, ERK activation is involved in the maintenance of high expression of $CD69^{+}CD4^{+}$ $CD25^{-}$ T cells via engagement of CD69. The subset is expanded in tumor-bearing hosts and may play a role in an inadequate response to tumors.

The role of $CD69^{+}CD4^{+}$ $CD25^{-}$ has been evaluated in leukemia relapse after allogeneic hematopoietic transplantation. The number of $CD69^{+}CD4^{+}$ $CD25^{-}$ T cells dramatically increases in patients in the relapsed group and the patients with positive minimal residual disease (MRD+) as compared to healthy controls. The therapeutic intervention results in the decrease of this subset. It seems that there is a correlation between $CD69^{+}CD4^{+}$ $CD25^{-}$ T cells and leukemia relapse after allogeneic hematopoietic transplantation. This suggests the need for adoptive T-cell immunotherapy.

Treg cells are present in high numbers in tumor tissues of lung, breast, pancreatic, gastrointestinal, and liver cancers and malignant melanoma. A poor prognosis for the breast, gastric, and ovarian cancers has been reported, if in the tumor-infiltrating lymphocytes there is a smaller ratio of $CD8^{+}$T cells to $FOXP3^{+}$ Treg cells and increased numbers of $CD4^{+}$ Treg cells. Based on these observations, $FOXP3^{+}$ Treg cells inhibit cytolytic T cells ($CD8^{+}$) that kill tumors. There is an improved prognosis for Hodgkin's lymphoma and head or neck and colon cancer, if there is an enhanced infiltration of $FOXP3^{+}$ Treg cells. These differing observations may be attributed to different makeups of $FOXP3^{+}$ Treg cell subsets in various tumors. This concept is supported by the reports that eTreg cells are present in larger numbers as opposed to naïve Treg cells and $FOXP3^{+}$ non-Treg cells in melanoma tumor-infiltrating lymphocytes, whereas in colon cancers there are high numbers of non-reg T cells and eTreg cells in infiltrating $FOXP3^{+}$T cells. A role of pro-inflammatory cytokines has been suggested for a better outcome in colon cancer, where there is an increased infiltration of $FOXP3^{+}$ non-Treg cells, despite possessing increased numbers of $FOXP3^{+}$ tumor-infiltrating lymphocytes. In cancer patients a variety of tumor molecules, such as Survivin and NY-ESO-1, are recognized by Treg cells, resulting in the inhibition of tumor-specific effector T-cell response.

$FOXP3^{+}CD4^{+}$ Treg cells express chemokine receptors. CCR4 attracts chemokine ligand 22 (CCL22) secreted by the tumor cells and/or tumor-infiltrating macrophages. Treg infiltration is also mediated by other combinations of chemokine ligands and chemokine receptors, including CCR10-CCL28 and CXCR and CXCL9, CXCL10, and CXCL11. It is not known whether regular T cells can become suppressive $FOXP3^{+}$ Treg cells in the microenvironment of human tumors. After reaching the tumor, $FOXP3^{+}CD4^{+}$ Treg cells go through induction and proliferation following recognizing tumor antigens or self-antigens, found due to tumor lysis. The natural $FOXP3^{+}CD4^{+}$ Treg cells, perhaps, are better in detecting tumor-associated self-antigens than tumor-reactive effector or memory $CD4^{+}$ T cells.

This is attributed to the fact that TCR repertoires are more self-reactive as compared to regular T cells. Furthermore FOXP3$^+$CD4$^+$ Treg cells exhibit enhanced expression of T-cell accessory molecules such as adhesion molecules (LFA-1). It has been suggested that cancer vaccines may activate tumor-specific Treg cells because of immunosuppressive tumor microenvironment and not tumor-specific effector T cells. Data obtained from clinical studies in melanoma and ovarian cancer patients have suggested that both healthy individuals, patients without cancer, and cancer patients possess T cells, which are potentially tumor reactive, but their induction and proliferation are inhibited by natural Treg cells. In these trials depletion of Treg cells resulted in the expansion of a tumor antigen-specific effector T cells from naïve T-cell precursors. These effector T cells harbored potent antitumor function.

CD-25, IL-2 receptor α chain and its high-affinity receptors, chemokine receptors, and other molecules predominantly expressed on Treg cells could be targeted for their depletion to treat cancer. The effector helper and cytolytic T cells express CD25, IL-2 secretion is required for their propagation, and inhibition of CD25 will result in the suppression of both the effector and Treg cells. Blocking CCR4 has been more promising. CCR4+ eTreg cells are in high numbers in the tumor-infiltrating FOXP3+ T cells in melanoma than those in the peripheral blood. As a result clinical trials are underway to assess the ability of anti-CCR4 monoclonal antibody to treat cancer because it induces and enhances antitumor responses. Another molecule of interest is glucocorticoid-induced TNF-receptor family-related protein (GITR), which is a costimulatory molecule with minimal expression on resting helper and cytolytic T cells. GITR is constitutively expressed with high density on FOXP3$^+$CD4$^+$ Treg cells. The use of agonistic anti-GITR monoclonal antibody or GITR ligand can antagonize the inhibitory function of FOXP3$^+$CD4$^+$ Treg cells. This also results in a loss of susceptibility of effector T cells to Treg cells. OX 40, another molecule, is transiently expressed on activated T cells but constitutively expressed on FOXP3$^+$CD4$^+$ Treg cells. The agonistic anti-OX40 monoclonal antibodies have shown antitumor effects by inhibiting FOXP3$^+$CD4$^+$ Treg cells and inducing effector T-cell function.

The induction of Fcϒ receptors (FcϒRs) enhances the effector function of lymphocytes and phagocytes that include antibody-dependent cell-mediated cytotoxicity and antibody-dependent phagocytosis, which in concert cause the removal of tumor cells. The mechanism behind these events involves the antibodies directed against OX40 (CD134). This molecule, OX40 (CD134), is a crucial costimulatory TNF receptor, which is transiently present on activated helper T cells (CD4$^+$). The antibodies to OX40 (CD134) remove intratumoral Treg cells in an activating FcϒR-dependent manner. These observations in a murine model are similar to other observations where Treg cells suppress TH1 effector responses in tumors. The success of checkpoint blockade inhibitors can be assessed by the presentation of TH1 immune polarity with a reduction in the ratio of intratumoral Tregs to effector T cells, mostly cytolytic T cells. It is intriguing that the monoclonal antibodies envisioned to act through inhibiting checkpoints or activating costimulatory molecules may simply be producing their pharmacologic responses through antibody-dependent cell-mediated cytotoxicity and antibody-dependent phagocytosis of Tregs, in

the tumor microenvironment. Antibodies directed against OX40 and other costimulatory molecules on T lymphocytes inhibit the immunosuppressive effects of Tregs and as a consequence augment the effector function of T cells against tumors. For these effects there seems to be a requirement of FcR-activating receptors and depletion of intratumoral Treg cells. However, immune response is not produced against many types of cancers and the role of Treg cells in these forms of cancers has not been established.

Tr1 cells also participate in a poor anticancer response. The infiltrating lymphocytes in Hodgkin's lymphoma contain both Treg and Tr1 cells, which suppress a variety of immune functions.

10.12.6 Transplantation

Treg cells play an important role in suppressing alloantigen-specific immune response following an organ graft. The maintenance of tolerance by various protocols in allograft transplantation is mediated via the induction of Treg cells in otherwise alloresponsive T cells. The responding T cells have a suppressive effect if naïve T cells are repetitively stimulated with immature allogeneic dendritic cells. The maximum T-cell response after allograft is dependent on the maturation stage of dendritic cells in the grafted tissue. Immature dendritic cells that result in suboptimal T-cell responses and limited costimulatory signals and cytokine production are ideal for the induction of Treg cells in responding T cells. In addition to the developmental stage, the particular subset of dendritic cells is also important in inducing Treg cells. Treg cells that are naturally occurring play an important role in the generation of induced Treg cells, resulting in a Tr1 suppressor phenotype from the graft-killing effector T cells. Furthermore, TGF-β can convert non-regulatory T cells to $CD4^+CD25^+$ suppressor cells. The tolerant grafts contain TGF-β and induce Treg cells in the graft, and there is also a presence of $CD8^+$ Treg cells in allografts. Patients undergoing allogeneic bone marrow transplantation who do not exhibit graft-versus-host disease have T cells that produce IL-10 and IFN-γ but low levels of IL-2.

10.13 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neuro-inflammatory disorder that destroys motor neurons resulting in death. Most of the patients diagnosed with the disease survive only 2–3 years while 10 % of the patients live about 5–10 years. The disease exhibits infiltrating lymphocytes which seem to be related to the enhanced chemokine CCL2 ligand levels and the morphological activation of microglia. A role of Treg cells has emerged as protectors of motor neuron as a result of their anti-inflammatory effects. In rapidly progressing patients, the numbers of

Treg cells and the expression of FoxP3 are reduced. Furthermore, there is inverse correlation between the disease progression and the number and expression of Treg cells and FoxP3, respectively. There is reduction in the mRNA levels of FoxP3, IL-4, TGF-β, and GATA3 in rapidly progressing patients with an inverse correlation. The patients who live longer have a higher number of regulatory T cells. It has been suggested that administration of low levels of IL-2 results in the production of Treg cells and a phase I/II randomized placebo-controlled clinical trial is planned to look into this further. In animal models of ALS, there is a change from the protective effects of Treg cells to injury-causing responses of effector T cells. The Treg cells switch the microglial responses from cytolytic effects to neuroprotection.

10.14 Future Direction

Recent understanding of Treg cells provides a number of unique opportunities for their use in therapeutic intervention. The expectation is that the therapeutic application of Treg cells will result in reestablishing tolerance, the breakdown of which has resulted in the development of the disease. The treatment of individuals with antigen-specific Treg cells will be an interesting approach. Treg cells will have several advantages including a long half-life, ability to condition other cell types, induction of non-regulatory cells to secrete IL-10, and suppressive effects on the costimulatory activity of antigen-presenting cells. However, so far the therapeutic use of Treg cells has been limited due to their low frequency in the circulating blood, and this will require industrial cultures for their propagation for clinical therapy. It has not yet been possible to use industrially produced cells therapeutically, since their culture conditions stimulate TH2 responses.

Development of culture techniques that have allowed the ability of continuous culture of $CD4^{+}CD25^{+}$ cells and the generation of these Treg cells from $CD25^{-}$ cells after FOXP3 gene is transduced by using a retroviral vector have permitted the production of these cells. A number of protocols have been published utilizing different stimuli for the culture of Treg cells on a large scale and their efficacy in in vivo models has been established. The use of $CD86^{hi}$ dendritic cells as antigen-presenting cells as compared with whole splenocytes is another approach in expanding ovalbumin-specific Treg cells. Furthermore, mature dendritic cells are a better stimulator than immature dendritic cells, and IL-2 or IL-15 secretion from dendritic cells is not required for their successful function. Since these cells remain dormant, unless stimulated, the choice of an expansion agent has been problematic. In various combinations four expansion agents, IL-2, anti-CD3, anti-CD3 plus anti-CD28, and rapamycin, are generally used. These expansion agents cannot be used therapeutically because of their toxicity. But they produce mixed population of CD4+ T cells, which cannot be used therapeutically due to the risk of the release of pro-inflammatory cytokines. Another approach has been to use TNFR2 agonist to produce phenotypically homogeneous population of Treg cells. Nonetheless, obtaining a homogeneous population of Treg cells in large enough numbers to be used as a therapeutic agent for treating a disease remains a challenge.

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Chapter 11
Gene Therapy

Abstract Once being part of the science fiction, the gene therapy is the delivery of a gene (nucleic acid polymers) to treat both inherited and acquired diseases. This is performed by using a transgene, which is the genetic information that a cell or tissue receives via a vector. A vector is used to carry the genetic information, and the type of transgene determines its potential outcome. This chapter describes the principles of gene therapy and the challenges associated with the procedure. There is a description of various vectors used to deliver the transgene and their advantages and disadvantages. The vectors commonly used include adenovirus, adeno-associated virus, retrovirus, lentivirus, herpes simplex virus 1, and vaccinia virus. In addition, RNAi- and RNA aptamer-based gene therapy is discussed. The major part of the chapter is devoted to the clinical/potential clinical use of gene therapy for a variety of inherited and acquired disorders. To date, the only procedure of gene therapy approved by the Food and Drug Administration is alipogene tiparvovec (Glybera) to treat lipoprotein lipase deficiency. Furthermore, the Food and Drug Administration has given LentiGlobin BB305 a "breakthrough" status for the treatment of β-thalassemia.

Keywords Gene defect • Lipoprotein lipase deficiency • β-Thalassemia • X-Linked ADA-SCID • X-Linked SCID • Retinal disease Leber's congenital amaurosis • Acute lymphocytic leukemia (ALL) • Chronic lymphocytic leukemia (CLL) • Multiple myeloma • Parkinson's disease • Hemophilia • Adrenoleukodystrophy • Transgene • Viral vectors • Adenoviruses • Coxsackie • Adenovirus receptor • α5 integrins • CAR • E1a • E1b • Virion • Adeno-associated virus • Integrins • Cystic fibrosis • AAV_1 • AAV_5 • AAV_6 • AAV_8 • Retroviruses • LTR • Gag • Env • Pol • Lentiviruses • Herpes simplex virus-1 vector • Vaccinia vectors • Amplicon-based vectors • Amplicon • Vibrotherapy • Nonviral vectors • RNAi gene therapy • Macular degeneration • Cancer • RNA aptamers • Macugen • Vascular endothelial growth factor • Alipogene tiparvovec • LentiGlobin BB305 • TRICOM • PANVAC-VF • PROSTVAC • Immunotherapy • Oncolytic virotherapy • Gene transfer • Leber's congenital amaurosis • Adrenoleukodystrophy • Neuralgene PRCN-323 • Ex vivo gene transfer • CFTR gene • Parkin gene • Dopamine • Substantia nigra • α-Synuclein • GTP cyclohydrolase I genes • Nigrostriatum • ProSavin • Vasculature • Cardiovascular disease • Ischemia • Angiogenic growth factor • Fibroblast growth factor • Atherosclerosis • Hypertension • Endothelial nitric oxide-synthase • Atrial

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natriuretic peptide • Thrombosis • Tissue plasminogen activator • Antistatin • Familial hypercholesterolemia • Human immunodeficiency virus • Ribozyme-based gene therapy • Epilepsy • LacZ marker gene • Neuropeptide Y • Galanin

11.1 Introduction

Although considered a modern therapeutic procedure, the theory of gene therapy goes back many decades. Historically, gene therapy has always been a part of science fiction with hope that its clinical applications will be realized. In the literature, these proposals go back many decades, and many have not yet been achieved. In ancient cultures, breeding plants and animals for certain goals was a way of life. Selective breeding practices were established before the development of modern genetics. The transfer of genes within DNA was first reported by Avery, Macloed, and McCarthy in 1944, and a few years later, it was observed that viruses possess the ability to transfer genes. On the basis of the ability of viruses to transfer genes, Tatum in 1966 hypothesized the role of viruses in gene therapy and the possibility of isolating or synthesizing genes and inserting them into the organs with defective genes. Aposhian in 1969 suggested the use of genes in place of drugs to treat diseases, and the initial gene therapy animal models included ducks and rats. The first gene therapy experiment in humans was performed by Rogers in 1969 to treat arginase deficiency, using Shope papilloma virus, but this was not successful. A significant advance in realizing this goal was achieved when Michael Wigler and Richard Axel in 1977 transferred a thymidine kinase gene into mammalian cells. Cline performed a gene therapy experiment in humans in 1980 to treat thalassemia, but his ethics was questioned and this resulted in his reprimand. In 1983, retroviral vectors were used to transfer a functional gene into cells followed by the development of helper-free retroviral packaging cell line. This resulted in the development of a number of gene transfer systems based on viruses. The foundation laid by this important work led to the first approved clinical trial of gene therapy in 1990 to treat ADA-deficient severe combined immunodeficiency.

The concept of gene therapy was originally based on treating diseases that were the result of a single gene defect. A defective gene causes abnormal protein synthesis or no synthesis at all, resulting in the etiology and pathogenesis of an inherited disease. The clinical manifestation could be mild or may be severe with a broad spectrum of abnormalities dependent on the importance of the protein in the physiologic processes. Providing the missing protein to the patient has always been a challenge due to the stability of the protein once it is administered. However, inserting a healthy gene that could synthesize the missing protein in the cell can overcome the challenge and help ameliorate the clinical symptoms of the disease. This scheme is plausible if the defective gene is expressed in only a few tissues. The genes that are expressed in all cells result in severe abnormalities, and generally, the pregnancy does not proceed. It entails using nucleic acid polymer as therapeutic agents for

curative treatment. After its delivery to the patients, they either get translated into proteins and fill a void or interfere with the function of other proteins and occasionally correct genetic mutations. The use of DNA that encodes a functional gene is the most common form of gene therapy. It is debatable that when gene therapy was originally conceptualized; however, the first FDA-approved gene therapy experiment in the United States was performed in 1990, when a patient was treated for severe combined immunodeficiency. To date, more than 2000 clinical trials have been performed, but it mostly remains an experimental procedure, and much work still needs to be done before its full potential would be realized. Gene therapy is generally used in somatic cells though the original vision was for the inherited disorder, which still is most crucial area of interest.

There has been success in using gene therapy for a variety of conditions. These include lipoprotein lipase deficiency, β-thalassemia, X-linked ADA-SCID, X-linked SCID, retinal disease Leber's congenital amaurosis, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma, Parkinson's disease, hemophilia, and adrenoleukodystrophy.

11.2 Altered Genes and Diseases of Inherited Disorders

Each individual often carries some defective genes, without any symptoms or disease. Almost 3000 diseases are known that are caused by an abnormal gene impacting about 10 % of the population starting from birth to any stage of life. The presence of a defective gene does not assure an abnormality in most individuals due to the presence of a second copy of the same (but healthy) gene with the exception of the genes on Y chromosome. However, in the case of dominant gene, the disease will be manifested although the other pair of the gene is normal and will require gene therapy. Under ideal circumstances, the insertion of a healthy gene should correct the problem; however, even calculating the amount of gene product that will provide the required levels of the lacking function protein is very tedious for some diseases. The strategy for gene therapy today is not only for the genetic disorders but also for the acquired disorders and as mediators of behavior, such as alcoholism and promiscuity, where these techniques are being used, at least experimentally, to treat disorders such as cardiovascular disease, cancer, and metabolic diseases.

Transgene is the genetic information that a cell or tissue receives via a vector that is used to carry this genetic information, and the type of transgene defines its expected therapeutic effect. There could be multiple reasons for which gene therapy is being performed; one is to compensate for a gene mutation where a healthy gene is provided for a missing or a mutated gene. The examples of the clinical trials have included the addition of a cystic fibrosis transmembrane conductance regulator gene in the lung epithelium and the restoration of p53 gene in cancer cells. The other type of gene therapy is purely therapeutic where the introduction of transgene causes the amelioration of disease pathology, such as in autoimmune diseases. Purely therapeutic gene therapy is also used for ischemic heart disease by introducing the vas-

cular endothelial growth factor gene that expresses the growth factor responsible for the development of blood vessels. An additional form of gene therapy uses transgenes, which are "suicide genes" and kill cancer cells after they are expressed in these cells. The examples of these genes include thymidine kinase, VSV-TK, deoxycytidine K and cytosine deaminase, proapoptotic genes, and enzyme prodrug combinations, such as HSV-TK and acyclovir, VSV-TK and Ara M, deoxycytidine K and Ara-C, and cytosine deaminase and 5-fluorocytidine. The suicide genes convert the prodrug to a toxic substance that kills the cancer cells, and the side effects could be controlled by limiting the doses of the prodrug. Lastly, gene transfer-mediated vaccination, which could potentially be used for both infectious and noninfectious diseases, is another approach for gene therapy. The example of noninfectious disease is to produce an immune response against the cancer cells with the induction of tumoricidal cytokines, tumor-infiltrating lymphocytes, and induced expression of lymphocyte costimulatory molecules, and the use of gene therapy instead of vaccination for infectious diseases may have therapeutic implications for AIDS. The various modes of gene delivery are depicted in Fig. 11.1.

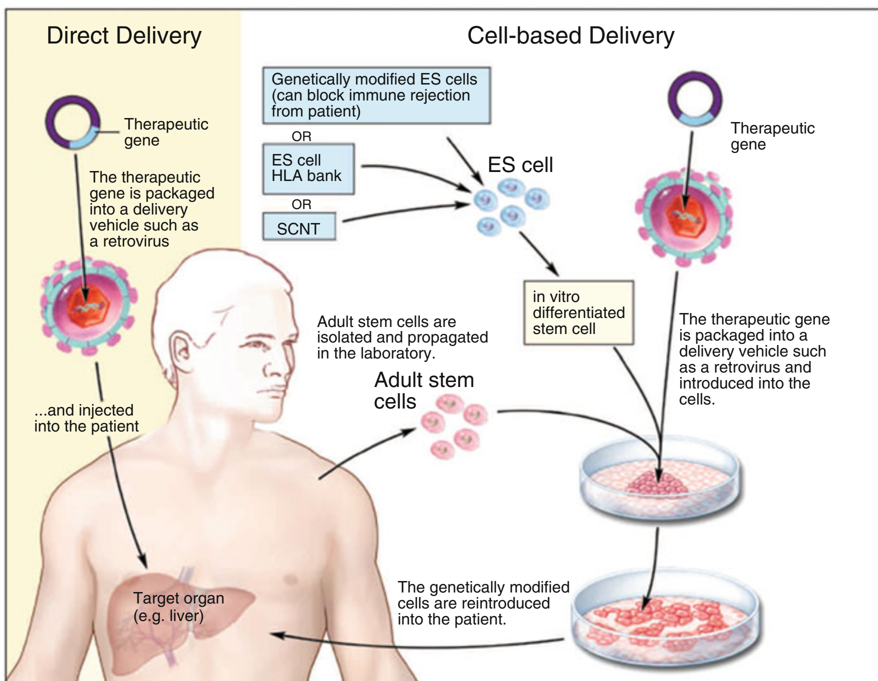

Fig. 11.1 Experimental gene therapy with genetically modified stem cells: gene therapy is possible by either living cells as vehicles or direct gene transfer into the recipient. This figure depicts the methods of delivering therapeutic transgenes into recipients via two methods (Source: NIH Public Domain. Author: Thomas P. Zawaka, http://stemcells.nih.gov/info/Regenerative_Medicine/Pages/2006Chapter4.aspx)

11.3 Vectors for Gene Therapy

The potential gene therapy vectors come in many different forms to deliver the genes, all of which have advantages and disadvantages. The priorities in a vector selection include its ability to deliver maximum levels of the gene, optimal expression of the gene for a desired period, and its ability to enter the target cell or tissue while sparing unintended tissues. A list of different gene transfer vectors used for gene therapy is provided in Table 11.1.

11.3.1 Adenoviruses

Adenoviral vectors are considered the most versatile and easily manipulated viral vectors for delivering genes, and over 50 identified serotypes of human adenovirus are known belonging to six species. They infect the respiratory tract in normal population, resulting in upper respiratory infection but their effects are self-limited.

Adenoviruses contain double-stranded DNA and are not enveloped and do not require the division of the host cells for their proliferation. They are ideal as a vector for gene therapy because they can penetrate a variety of tissues including respiratory epithelium, cardiac and skeletal muscle, vascular endothelium, hepatocytes, peripheral and central nervous system, and a number of different tumors. The ability to infect various tissues is dependent on the presence of Coxsackie and adenovirus receptor (CAR) and α5 integrins. This could be a potential problem since certain tissues and forms of cancer that could benefit from gene therapy are not rich in expressing CAR required for optimal infection with the adenovirus. The modification of proteins present on the capsid of adenovirus, which interact with CAR results in changing the specificity of adenoviruses for the target tissue – allowing targeted delivery and avoiding entry into nontarget tissue. Infectivity could be increased by

Table 11.1 Gene transfer vectors

Viral	Nonviral
Adenovirus	Lipoplexes
Adeno-associated virus	Polyplex
Retrovirus	Oligonucleotides Antisense RNA SIRAN RNA/DNA chimera Aptamer Ribozyme
Lentivirus	Naked DNA
Herpes simplex virus-1	Lipoplexes
Vaccinia virus	Cationic lipids
Amplicon based	Molecular conjugates

replacing existing knob proteins and adding different knob proteins from different adenovirus serotypes, other motifs, or single-chain antibodies. This allows targeting the tissues that may not previously contain abundant receptors for targeting and provides an opportunity for tissue targeted delivery.

Adenoviruses are ubiquitous as most adults are exposed to them, and their rearrangement is not observed at a high rate resulting in the stability of inserted genes after several cycles of viral replication. They contain 36 Kb of double-stranded DNA. After their entry into the host cell, E1a and E1b genes, which are the genes from the early region, are transcribed. There is an expression of E_1, E_2, E_3, and E_4 genes as the virus replicates, and these genes regulate transcription, which leads to genome expression. Most of the viral transcription is regulated by the late promoter after DNA replication is initiated. There are two types of elements, Cis and Trans, which perform separate viral functions. The virus itself, with the help of Cis genes, is responsible for the replication and condensing of DNA. The Trans genes can be removed or helped by other inserted genes. The foreign DNA can be placed in one of the three regions, which include E_1, E_3, and a small portion of E_4. Immunogenicity of the adenovirus can be reduced by removing various E_1, E_2, and E_4 genes, resulting in lower toxicity to the host. However, this viral redesign does not reduce MHC Class-II-dependent T-helper responses.

Adenoviral vectors have been modified to create vectors, which do not contain all viral protein-coding DNA sequences. This is a helper-dependent vector system in which all the viral genes required for replication are present, but its packaging domain has a deficiency, resulting in its inability to form a virion. Its combination, a second vector, possesses therapeutic genes as well as normal packaging recognition signal, which allows its genome to form a virion and released. This vector system is also called "gutless" adenoviral vectors. These viruses can be delivered by a wide variety of modes of administration including intravenous, intraperitoneal, intrabiliary, intracranial, intrathecal, and/or intravesicular injections.

Adenoviruses are used for the treatment of cystic fibrosis and cancer by gene therapy. For the treatment of cystic fibrosis, aerosolization has been used to deliver the gene for cystic fibrosis transmembrane conductance regulator that codes for a transmembrane cAMP-regulated Cl^- channel on the epithelial cell surface. However, many hurdles still exist in this therapeutic procedure.

Various protocols have been used to treat cancer by gene therapy using adenoviruses, which include gene-based immunotherapy, prodrug therapy, and gene replacement approaches. These procedures are often employed in combination with standard chemotherapy. One example of gene-based immunotherapy to treat cancer is the use of adenoviruses to augment normal B-cell and T-cell responses against tumor-associated antigens. Another example involves transducing autologous tumor cells to make them produce GM-CSF, which has been used to treat malignant melanoma. A different approach that has been experimentally used to treat brain tumors utilizes prodrug therapy in which gene therapy using adenoviruses is based on delivering genes for enzymes into tumor cells, which then convert prodrugs to cytotoxic chemotherapeutic agents. Other cancer gene therapy protocols are based on the observations that tumor cells are more responsive to conventional chemotherapy if

they express wild-type p53 molecules that are associated with apoptosis, and interference with this function results in resistance to chemotherapy by tumor cells. Gene therapy could be employed to remedy defects in p53 by reestablishing apoptosis-inducing regulators. Alternatively, genes which induce apoptosis such as tumor necrosis factor α, caspase-8, and FAS ligand can be added.

11.3.2 Adeno-Associated Virus

Adeno-associated virus was named because it is often found in cells that are also infected with adenovirus. This non-enveloped, nonautonomous parvovirus has been well studied for gene therapy, has single-stranded DNA, and can integrate into the genome at chromosome 19 of non-dividing cells. Adeno-associated virus naturally expresses transgenes for an extended period as opposed to adenovirus and has multiple serotypes that enables the virus to infect a wide variety of tissues. The virus is not associated with a human disease, and the infected host does not generate either antibodies or an inflammatory response against it. Adeno-associated virus is attractive for gene therapy because it lacks pathogenicity, can infect non-dividing cells, and stably integrates into host cell genome at a specific site, and its ability to integrate only at chromosome 19 makes it superior to other viruses, which by random insertion and mutation may cause cancer. Its disadvantages are that it has a limited transgene capacity, and two vectors are needed simultaneously to appropriately express the transgene. The virus uses integrins as well as heparin sulfate proteoglycans as a primary receptor and fibroblast growth factor 1 as a co-receptor 1 for infecting the host cell.

Adeno-associated virus transgene has been tested as a delivery system for cystic fibrosis where AAV serotype 2 was used in clinical trials since this serotype infects lung epithelium. Other serotypes being tested for gene therapy include AAV_1, AAV_5, AAV_6, and AAV_8. AAV_1 and AAV_5 are efficient in delivering genes to vascular endothelial cells, AAV_6 has been more effective than AAV_2 in infecting airway epithelial cells, and AAV_8 is very effective in transducing hepatocytes.

11.3.3 Retroviruses

Retroviruses present themselves with an advantage over adenovirus and AAV vectors due to their ability to integrate the transgene DNA into the host and for their potential of long-term expression, which make them a preferable candidate for gene therapy specifically for curative treatments. They are not immunogenic, and the host lacks preexisting antibodies or T cells against them. Despite these advantages, undesirable side effects always pose a risk due to their ability to integrate into host cells permanently. Furthermore, their application for gene therapy could only be limited to the dividing cells. In SCID trials where retroviruses were used as vectors

for gene therapy, the children developed leukemia, which was attributed to proliferation of T cells as a result of mutation.

Retroviruses were first used for gene therapy in 1989. They contain two copies of RNA genome and are packaged in an envelope that is like a cell membrane. The envelope fuses with the host cell membrane in some retroviruses, whereas it is endocytosed in others. The positive-strand RNA genome is transcribed to cDNA by the reverse transcriptase contained in the viral envelope, and cDNA then migrates into the nucleus followed by integration into the host's cell DNA. This process is mediated by viral integrase, which is also present in the envelope of the retrovirus. The viral mRNA is transcribed by the integrated provirus, which is subsequently processed and translated into viral proteins. The integrated provirus synthesizes a positive-strand RNA genome, which is then packaged with other proteins, and particles are released from the host cell via a process called budding.

A retroviral genome is composed of 5^- and 3^- long terminal repeats (LTR), a group-specific antigen gene (gag), reverse transcriptase (pol), and envelope protein (env). Furthermore, the psi sequence and a Cis-acting element are critical for packaging. Without a psi sequence, RNA cannot be packaged.

The most commonly used retroviruses for gene therapy are the murine leukemia viruses. The proviral form of the virus is used to construct retroviral vectors. A standard protocol involves the removal of gag, env, and pol genes, which are responsible for the replication of the virus. Furthermore, this deletion makes way for the space where the therapeutic gene will be placed. Retroviruses can accommodate up to 8 kilobases of therapeutic genes. This retrovirus will not synthesize any viral proteins, resulting in protection from any form of immune response which may result if viral antigens were produced. Once the genes encoding viral structural proteins are deleted, special viral packages or packaging cell lines require insertion of the deleted genes; however, this is done on a different chromosome; thus, these genes will not be able to package with the vector. This system allows the production of gene-deleted vector at a large scale, which can be used to insert the therapeutic genes and then used for gene therapy. The recombinant proviral DNA is inserted in the package system to produce the recombinant vector. Many different techniques have been used to insert the recombinant gene into the packaging cell line.

The recombinant vector can now be administered in the patient by either the ex vivo transduction or by directly injecting the virus into the patient's target tissue. Alternatively, the retrovirus-producing cell can also be injected. However, the most commonly used technique in clinical trials has been the ex vivo transduction of patient's cells, which allows quantification of gene transfer and targeting of the specific cell population. The transduction efficiency is further improved by employing this technique since it allows the use of a high ratio of viral particles to target cells.

As opposed to the tissues that are easily accessible, others present a challenge for delivery, such as brain tumors. Retrovirus provides a unique opportunity to treat cancer because of their ability to transduce only the dividing cells. In experimental models, T cells have been used to successfully deliver retrovirus to metastatic tumor deposits in the lung and liver. T cells have also been engineered to produce retroviral vectors using rapamycin induction. Sirolimus has been used because it mediates

heterodimer formation between the FK-binding protein and lipid kinase FRAP, and by fusing FKBP domains to DNA-binding domain and FRAP to a transcriptional activation domain, assembly of an active transcription factor and expression of a target gene can be made dependent on the presence of sirolimus.

The disadvantages of retroviruses include the limited number of cells that are transduced by virus due to dilute retrovirus preparations and their ability to only transduce the dividing cells. To overcome these problems, retrovirus-producing cell lines are directly administered into the tumors. Although there has not been any report from the clinical trials, the concerns regarding the mutagenicity after integration of the retrovirus into the host genome have been of concern.

11.3.4 Lentiviruses

Lentiviruses are a subclass of retroviruses and can integrate both into the dividing and non-dividing cells. Despite the rapid evolution rate of these viruses, many elements in their genome have been conserved over time. The gag and pol genes are conserved, but the env gene is much more variable. After the virus enters the cell, its genome, which is RNA, undergoes transcription to DNA by its reverse transcriptase and a viral integrase randomly incorporates it into the host genome. As a result, a provirus is formed and will propagate with the proliferation of the host cell. The provirus may interfere with the normal functioning of the host cells and even could induce oncogenes. For gene therapy, lentivirus vector is modified to remove the genes needed for replication. Lentivirus is produced by transfecting several plasmids into a packaging cell line, which include packaging plasmids encoding the virion proteins and genetic material that the vector will deliver. The single-stranded RNA viral genome is used to package the genome into the virion.

Lentiviruses can be very effective vectors for gene therapy since they can change the expression of genes in target cells for up to 6 months. They are useful for non-dividing and terminally differentiated cells including muscle cells, hepatocytes, neurons, macrophages, retinal photoreceptors, and hematopoietic stem cells. However, lentiviruses cannot enter quiescent cells in which reverse transcription is blocked.

11.3.5 Herpes Simplex Virus-1 Vector

Herpes simplex virus-1 (HSV-1) vector is a human neurotrophic vector that possesses double-stranded DNA, replicates in the nucleus of the infected cells, and can infect both dividing and non-dividing cells. It could also live in a nonintegrated state. The virus has received attention as a vector for gene transfer in the nervous system, since HSV-1 infects neurons and may enter a lytic life cycle or live as an intranuclear episome, which is a latent state. The latent virus does not replicate

despite the presence of active neuron-specific promoters, and an immune response is not produced against the latently infected neurons.

Viral genome can be inserted with a large DNA sequence by homologous recombination, and the recombinant virus, which is defective in replication, can be plaque purified by using transcomplementing cells. However, this viral vector has several disadvantages: it is difficult to obtain preparations that are completely defective in replication, and the modified vector generates immune response and antibodies specific for HSV-1 are present.

11.3.6 Vaccinia Vectors

Vaccinia virus, a member of pox virus family, has been used for small pox vaccination and for other infectious agents. They are large enveloped viruses with DNA as genetic material, a large genome of 130–200 kilobases, and can infect a wide variety of cell types including both the dividing and non-dividing cells. As nonintegrated genome, their gene expression is for a short period. The recombinant virus is produced by homologous recombination, and it requires a viral vector DNA and a packaging cell line, as large genome allows the insertion of large genes. An immune response is produced against almost 200 antigens of the virus, although the adverse reactions are not that common, but its use in gene therapy has to be limited to individuals who have not previously received small pox vaccination. This system has the potential to allow for the vaccination against diseases that are not currently treatable including HIV infection. The main concern with this vector in addition to its immunogenicity is of its replication, specifically in immunodeficient patients. Vaccinia vector has been used for clinical trials for cancer where it is expected to directly stimulate the immune response to destroy cancer cells. A summary of the characteristics of the above described viral vectors used for gene therapy is depicted in Table 11.2.

Table 11.2 Some viral vectors used for gene therapy

Vector	Packaging capacity	Titer	Integration	Expression	Tropism	Transduction efficiency
Adenovirus	8 KB	10^{11}	No	Transient	Broad	High
Adeno-associated virus	<5 KB	10^{9}	Yes (?)	Transient	Broad except for hematopoietic cells	High
Retrovirus	8 KB	10^{7}–10^{10}	Yes	Variable	Only dividing cells	Low
HSV-1	40–150 KB	10^{8}	No	Transient except neurons	Strong for neurons	High
Vaccinia	35 KB maximum	N/A	No	Transient	Broad	High

11.3.7 Amplicon-Based Vectors

Herpes simplex virus amplicons are plasmids that contain HSV replication origin and packaging sequence. These vectors lack parts of HSV genome that is required for replication and depend upon naturally occurring defective interfering particles (DI vectors), which are infectious agents formed during HSV propagation. Plasmid DNA containing HSV packaging signal can be packaged into defective interfering particles. After the insertion of the gene into amplicon, it is transfected into a cell line that is appropriate for HSV proliferation, which is followed by the infection of the cells with helper HSV virus. The proliferation of the virus results in the production of DI vectors and helper viruses. However, the use of amplicon-based vectors for gene therapy has been restricted because of the inability to purify the DI vectors that are not contaminated with helper virus. Nonetheless, HSV amplicon-based factors allow genetic transfer of multiple transgene copies in the absence of viral genes. Consequently, due to their relative flexibility, as opposed to other viral vector systems, they are used in clinical trials for cancer gene therapy as part of oncolytic vibrotherapy and immunotherapy. The advantages and disadvantages of various viral vectors used in gene therapy are described in Table 11.3.

11.3.8 Nonviral Gene Therapy Vectors

The procedures for nonviral vectors in gene delivery include physical methods such as naked DNA delivery by ultrasound or electroporation or endocytosis-mediated mechanisms. These protocols provide alternative to the viral vectors for gene

Table 11.3 Advantages and disadvantages of various viral vectors

Vector	Genetic material	Advantages	Disadvantages
Adenovirus	dsDNA	Excellent transduction of most tissues	Potent inflammatory response against the virus
Adeno-associated virus	ssDNA	No toxicity. No inflammatory response	Limited packaging capacity
Retrovirus	RNA	Transduction efficiency is high. Sustained expression of vector after integrating into host genome. Host does not express vector proteins	Dividing cells are required for infectivity. Random integration. Oncogenesis may be induced after integration
HSV-1	dsDNA	Large packaging capacity. Strong tropism for neurons	Transgene expression is transient except neurons. Inflammatory response against the virus
Vaccinia	dsDNA	Allows insertion of large genes. Minimum adverse reactions. Anticancer effects of immunogenicity	Immunogenic. Limited to non-smallpox-vaccinated or -immunodeficient subjects

delivery, but these methods are not as efficient as viral vectors because of method of entry and DNA degradation in the endosomes or lysosomes. However, limited success has been reported by the use of liposome for delivering genes to treat cancer. In these experiments, adenovirus mu protein or protamine sulfate were used for protecting the DNA degradation. One of the drawbacks of cationic liposomes is their limited ability in delivering transgene to the target cells and the induction of its expression.

Other vehicles under development for nonviral gene delivery are polymers that are positively charged and are filled with large negatively charged DNA to facilitate endocytosis. The targeting is further improved by the incorporation of ligands into polymers. In some studies, folate and transferrin have been used for receptor-mediated endocytosis. In other cases, biodegradable polymers slowly release transgene around the cells as they degrade, which increases the transfection efficiency and also provide extended expression of the gene. This efficiency has been improved by the use of polymers, such as polyethylene glycol, against which an immune response is not produced.

Various strategies have been developed to overcome difficulties associated with gene delivery when using nonviral vectors. Some of these examples include internalization of the carrier, polynucleotide degradation in the extracellular space, dissociation of the polynucleotide from the carrier, entry of the polynucleotide into the nucleus, and intracellular trafficking from endosome to lysosome. The focus has been the development of multifunctional nonviral vectors that could penetrate different barriers. The major parts of such a system are molecules, such as protamine and polyethylamine, which not only can condense polynucleotides but also can protect against nucleases when injected into the blood DNA–cationic carrier complex aggregate. However, this positive charge could be protected by PEGylation of the cationic carrier that enhances blood circulations and impedes aggregation. The PEGylated nanocarriers home in richly vasculated tissue, and if ligands are then attached to the distal end of the PEG to induce receptor–mediator endocytosis following the cell surface recognition, the cellular uptake is significantly augmented. The encapsulated polynucleotide is released in the cell following endosome rupture caused by endosomolytic activity contained in the components of the carrier.

While most of the studies using nonviral vectors are being performed in animal models, O'Malley et al. have performed IL-2 gene therapy by using nonviral vector in patients with advanced head and neck cancer. In their phase I clinical trial, cationic liposomes prepared with CMV-IL-2 plasmid were injected into the tumor. All patients completed the study but most died due to the progression of the disease. One patient exhibited a decrease in the burden of the tumor.

11.3.9 RNA Interference Gene Therapy

RNA interference is a technique where a strand of RNA in a cell destroys another RNA strand. This second strand of RNA is responsible for relaying protein coded messages from a gene, and its destruction means that the gene's message could no longer be carried out. The rationale for using RNAi-based gene therapy is to

knockout genes that are overexpressed in diseases such as macular degeneration or cancer. For the treatment of viral infections, RNAi will disable the gene required for the survival of the virus, and the expectation is that this technique will shut off genes for viruses including HIV and hepatitis, and consequently the disease will not result, due to the inability of these viruses to replicate. The drawbacks of this technique have been the production of immune response and inadvertent switching off of the wrong genes, resulting in unexpected toxic effects. Human RNAi-based gene therapy trials have been conducted and several drugs are in pipeline to treat various diseases, including liver disease and cancer.

11.3.10 Gene Therapy Using RNA Aptamers

Aptamers are small structured single-stranded RNAs or DNAs that have been produced by using a technique called systematic evolution of ligands by exponential enrichment (SELEX). Aptamers recognize and bind their cognate ligands utilizing complementary three-dimensional structures. SELEX consists of selection and amplification phases that reduce library of the nucleic acid, and the final product is made up of one or more sequences of nucleic acids purified from complex randomized sequences with affinity for a specific target. After binding, aptamers become encapsulated by the ligand, and the target gets incorporated into the intrinsic structure of the aptamer. Aptamers are distinct as they are three-dimensional globular structures, as opposed to ribozymes and antisense oligonucleotides that are linear and act at mRNA levels to alter protein expression. They are specific molecules with high affinity for the target that acts like antagonists by binding to the functionally relevant part of the protein. The stability and bioavailability of aptamers can be augmented by simple chemical modification. They do not produce an immune response and lack any toxic effects. Their clinical use was initially limited due to poor availability, limited cellular delivery, rapid clearance, and sensitivity to nucleases, but many of these issues have been resolved with new preparations of aptamers.

The first aptamer drug, Macugen, was approved for the treatment of wet-age-related macular degeneration (AMD); it is an antagonist of vascular endothelial growth factor and binds to the heparin-binding domain. Additional aptamers under clinical developmental stages include transcriptional decoys and thrombin-specific aptamers. Transcriptional decoys are made up of small RNAs or double-stranded DNAs and contain a specific transcription factor's consensus binding sequence. The inhibition of gene expression is achieved after a decoy binds to its transcription factor's site, resulting in the prevention of the binding of the protein to the target gene's promoter region. Several transcriptional decoys are under development for cardiovascular diseases, inflammation, arthritis, liver disease, and dermatitis.

Other aptamers under clinical trials are for coagulation factors including thrombin, Factor VIIA, Factor IXA, and Factor XII. Additional aptamers are being tested for kidney and lung cancer. A distinct advantage of aptamers is the availability of antidotes for them, which are short complementary sequences to the aptamers that serve as their antagonists and could be used to avert aptamer toxicity.

For the long-term gene therapy against HIV, focus has been placed on combining RNA-based therapies including aptamers (decoy RNA), siRNA, and ribozyme as their combination helps overcome the shortcomings associated with each form of therapy. Furthermore, distinct phases of the HIV cell cycle can be targeted by using specific combination of RNA-based therapies. An example is a lentiviral-based construct that contains transgenes for both a CCR5 ribozyme and a U16TAR decoy. CCR5 ribozyme is a downregulator of HIV-1 co-receptor, and U16TAR decoy is an inhibitor of Tat-activation transcription in HIV. The primary $CD34^{+}$-derived monocytes possessing this vector exhibit strong resistance against HIV. Another example is a vector combining three forms of RNA-based therapies that include a U6 transcribed nucleolar-localizing TAR RNA decoy, a U6 Pol III promoter-driven short hairpin RNA, and a VA1-derived Pol III cassette that is effective in inhibiting the proliferation of HIV.

11.4 Treatment of Diseases by Gene Therapy

11.4.1 Lipoprotein Lipase Deficiency

11.4.1.1 Alipogene Tiparvovec (Glybera)

Alipogene tiparvovec is the first gene therapy approved and is the treatment for familial lipoprotein lipase deficiency. This deficiency results from a mutation in a gene that codes for lipoprotein lipase. It affects about 1 out of 1,000,000 individuals. Lipoprotein lipase is required for the processing and clearance of fat-carrying chylomicron particles. These particles are made in the intestine after a fatty meal. The deficiency can cause severe pancreatitis. An intact copy of human lipoprotein lipase gene is delivered using the adeno-associated virus serotype 1 (AAV1) viral vector. The vector contains the gene packaged with a tissue-specific promoter. It has particular affinity for the muscle cell, which allows the muscle cell to produce the enzyme. This vector (AAV1) lacks pathogenicity and is delivered to the non-dividing cells. It is not very immunogenic, and there is production of only some neutralizing immunoglobulins and cytolytic T cells. Genetic testing and suffering from multiple or severe pancreatitis attacks despite dietary fat restrictions are the requirements, before the administration of the therapy. Alipogene tiparvovec is administered in a one-time series of up to 60 intramuscular injections in the leg. The procedure is performed under deep sedation. There is a recommendation of prescribing immunosuppressive agent/s beginning 3 weeks before and ending 12 days after the conclusion of alipogene tiparvovec therapy. According to the reports, the fat concentration in the blood is reduced nearly in all the patients, which takes about 3–12 weeks after the injection of the gene carrying vector. There is also a reduction of pancreatitis attacks in some patients. The most common side effects are pain in the legs following the injections. Other common side effects include headache, fatigue, bruising, and hyperthermia. It is contraindicated in patients with muscle disease, immunodeficiency, and increased risk of bleeding. The patients should also not take oral contraceptives.

11.4.2 B-Thalassemia

11.4.2.1 LentiGlobin BB305

LentiGlobin BB305 is an experimental gene therapy procedure to treat β-thalassemia. This disease is caused by deletion or mutation of the HBB gene, resulting in a decreased or lack of synthesis of the β-chains of hemoglobin. This will result in clinically asymptomatic individuals or patients with severe anemia. LentiGlobin BB305 is a lentivirus-based gene therapy drug that contains an operational HBB gene. The transgene vector is transduced into human hematopoietic stem cells in vitro. These engineered cells are administered to the patient with β-thalassemia. The Food and Drug Administration has given "breakthrough" status for this treatment on February 2015.

11.4.3 Cancer

Gene therapy is a broad field, and for cancer, it can be divided into three distinct forms of treatment: (a) immunotherapy, (b) oncolytic vibrotherapy, and (c) gene transfer. The concept of immunotherapy to treat cancer, where an augmented host response against the tumor cells is intended, is not a new one. A major current focus of this approach is to develop recombinant cancer vaccines that could be employed to treat and not prevent the disease. This is being done by the administration of the immunostimulatory genes, which after entering the tumor cells produce proteins resulting in a production of humoral and cellular responses against the tumor cells.

Another type of protocol is used is to alter the immune response of the patient, resulting in sensitization against cancer cells. In this form of gene therapy, a tumor antigen or a stimulatory gene is added to the peripheral blood lymphocytes or bone marrow cells of the patient that are then primed to produce an immune response against the cancer cells. Initial trials have been performed for cancer treatment by using first-generation vaccines but have provided mixed results. Nevertheless, these trials have allowed a better understanding for developing the next generation of cancer vaccines. A number of next-generation vaccines are under trial for various types of cancer. GVAX, a vaccine produced by modifying autologous tumor cells to express GM-CSF, has been used alone and in combination with cyclophosphamide to treat lung cancer, and the results have been promising. In other studies the immunostimulatory genes are directly inserted in the tumor cells as has been the case for clinical trials for malignant melanoma patients. In this specific case, genes for IL-24 have been inserted into the tumor cells and adenovirus has been used as the vector. Other vaccines which are undergoing clinical trials include TRICOM, PANVAC-VF, and PROSTVAC. In TRICOM vaccines a cancer antigen is added to vaccinia vector, where genes for ICAM-1, B7-1, and LFA-3 have also been included. PANVAC vaccine is for pancreatic cancer in which a modified vaccinia virus has been used as a vector that was supplemented with genes for immunostimulation, CEA and MUC-1. This vaccine is injected subcutaneously, and booster vaccine using a fowl pox virus that is modified similarly to the vaccinia virus is subsequently administered.

Another form of gene therapy to treat cancer involves oncolytic virotherapy. This involves the use of oncolytic vectors, which are viruses designed to home and kill the tumor cells without harming the normal cells in the body. The cancer cells are killed by cell lysis as a result of the production of cytotoxic proteins or due to the propagation of the virus itself. The viruses that have been used to produce oncolytic vectors include adenovirus, vaccinia virus, retrovirus, herpes simplex virus-1, and Newcastle disease virus.

An adenovirus vector, ONYX-015, which lacks E1B protein that is required for replication with a normal P53 pathway and RNA export during viral replication, has been used to treat squamous cell carcinoma of the head and neck and has also been tested as a preventive treatment for oral precancerous tissue. The concept behind using this vector is that ONYX-015 will proliferate in p53 pathway-deficient tumor cells and kill them.

Two other vectors that have been used clinically are G207 and NV1020, both of which are modified herpes simplex virus-1. They have been modified, such that they could successfully replicate only in tumor cells. G207 cannot proliferate in non-dividing cells and has high affinity for neurons. The mutations in NV1020 include insertion of thymidine kinase gene controlled by α4 promoter, deletions in several components of the genome, and a deletion in the thymidine kinase region. These vectors are tested for cancer gene therapy in treating malignant glioma (G207) and for colorectal and liver cancer (NV1020). They utilize two mechanisms to kill cancer cells: (a) lytic portion of life cycle directly causes killing and (b) thymidine kinase produced by the virus makes tumor cells susceptible to ganciclovir.

The third type of gene therapy for cancer treatment is gene insertion. These genes include suicide genes, cellular stasis genes, and antiangiogenesis genes. The gene delivery systems used are both viral vectors and nonviral systems such as DNA transfer, oligodendroma DNA coatings, and electroporation. The choice depends on the need for specificity or period of desired expression of the gene. Avoiding the incorporation of these genes into the unintended target cells is the real challenge. The early experiments encountered the problem of gene silencing, where the inserted gene was either not expressed or its expression was very limited. Some examples of this form of gene therapy include TNFerade, Rexin-G, and adenovirus delivering the HSVtk gene. TNFerade is a modified adenovirus-based vector that delivers the gene for TNF-α and is under the control of a promoter that is induced by radiation, which necessitates receiving radiation for the patient after this vector is injected. The combination of radiation and TNF-α kills the tumor cells, and this form of gene therapy has been tested for the treatment of melanoma, rectal cancer, pancreatic cancer, and esophageal cancer. Rexin-G is a retroviral-based vector that delivers a gene that interferes with the function of cyclin G_1 gene. This gene after entering into tumor cells disrupts the cell cycle and has been tested for pancreatic and colon cancers that have spread to the liver.

Cell-targeted suicide is another form of gene therapy, which is achieved by delivering a gene. Insertion of HSV-thymidine kinase gene by using adenovirus vector into malignant cells in conjunction with the systemic administration of ganciclovir has gone through clinical trials to treat cancer. Ganciclovir is not toxic before it is

metabolized by the enzyme. Consequently, the treatment would affect only the cells that have received the HSV-thymidine kinase gene. This treatment has been tested for patients with glioblastoma.

Other approaches involve the delivery of p53 gene into tumor cells by using adenoviral vector. A mutation in p53 gene results in some forms of cancer, and providing a healthy copy of the gene may stop proliferation of the tumor cells and also cause apoptosis. INGN201 has been used as a carrier of p53 gene for gene therapy in patients with squamous cell carcinoma of the head and neck, glioma, bladder cancer, ovarian cancer, and prostate cancer.

11.4.4 Gene Therapy for Human Retina

Retinal gene therapy appears to be promising to ameliorate both inherited and non-inherited blindness. Gene therapy has been successful in treating a rare genetic retinal disease, Leber's congenital amaurosis. In these studies adeno-associated virus (AAV) vector is utilized to deliver a functional copy of the RPE65 gene. The gene is delivered by "subretinal injection." This treatment provided vision to children suffering with Leber's congenital amaurosis. There is improvement in visual function in all patients receiving the gene. The criteria assessed to measure the sight include functional mobility, visual acuity, improvements on functional MRI, and pupil's ability to respond to light. These patients retain their improved visual function after more than one and a half years. These observations lend credibility to the use of adeno-associated virus vector in treating other forms of retinal diseases. The advantage of this vector in treating retinal diseases is it not being immunogenic and its long-lasting transgene expression. In the retina, the protection is provided from invading pathogens by high junctions that constitute the blood–retina barrier and spares the subretinal space from the blood supply. This suppresses retinal damage, resulting from the immune response.

Similar treatments have been under clinical trials for the treatment of age-related macular degeneration, choroideremia, and color blindness, using adeno-associated virus as a vector. The results from the phase1/2 trials have demonstrated the restoration of REP gene in patients with choroideremia, after the gene is delivered using subretinal adeno-associated virus.

11.4.5 Adrenoleukodystrophy

Adrenoleukodystrophy, also known as X-linked adrenoleukodystrophy or adreno-myeloneuropathy, is an abnormality of peroxisomal fatty acid beta oxidation. This results in the deposits of very-long-chain fatty acids throughout the body. Some tissues are more affected than other. The most adversely affected tissues include the adrenal cortex and the myelin in the central nervous system. The disease exhibits a

deficiency in adrenalin (known as Addison's disease), cognitive decline mood changes, cognitive decline, and dementia. Adrenoleukodystrophy is a result of mutations in ABCD1, a gene located on X chromosome coding for adrenoleukodystrophy, a peroxisomal membrane transporter protein. It is a common inherited disorder with an incidence of 1:20,000 and 1:50,000. The disease is more prevalent in males than females. Only 50 % of the heterozygote women exhibit some symptoms in later part of life. The most severe form of the disease is observed in early childhood, where normal development rapidly turns into degeneration causing a vegetative state. For gene therapy, the vectors are modified to express wild-type ABCD1 that afterward is transplanted into the patient by using a protocol of bone marrow or stem cell transplant. In a clinical trial in France, gene therapy reversed the symptoms of adrenoleukodystrophy but has no effect on the levels of very-long-chain fatty acids. In another study, Neuralgene PRCN-323 has been used to treat one patient. For this gene therapy-based drug, adeno-associated virus is used as a vector to deliver a normal copy of ABCD1 gene. The transgene containing vector is administered into the spinal fluid. The complete results of this trial are not available at this time.

11.4.6 Cystic Fibrosis

Gene therapy has presented itself as an attractive option for treating cystic fibrosis over the decades. Most of the morbidity and mortality in patients with cystic fibrosis result from pulmonary dysfunction, and this is the most common genetic disorder among Caucasians. The lung is not suited for ex vivo gene transfer methods, and the removal and regrafting of the airway cells is not practical. The organization of the cystic fibrosis transmembrane conductance regulator gene and its mutations causing the pathophysiological symptoms are now better understood.

A number of clinical trials have been performed to assess the potential of gene therapy to treat cystic fibrosis. The data obtained from using topical cystic fibrosis transmembrane conductance regulator gene therapy is not promising. One would have expected that insertion of a normal copy of the CFTR gene into the patient's cells, in the affected epithelium, will cause the synthesis of normal CFTR in the desired cells. It is estimated that only 5–10 % of the normal expression of CFTR gene would suffice. Many of the vectors already described have been tested in clinical trials with limited success. The major problem has been the inability of successfully delivering the transgene vector into the desired cell type. This has been attributed to the low expression or absence of cellular receptors and co-receptors, which are required for viral binding and entry into the target cell. As a consequence, there is minimal gene expression and amelioration of symptoms of cystic fibrosis. There are also other issues, such as difficulty with cDNA recombination, which causes the delivered gene to be of no use. However, there has been a functional repair of CFTR by CRISPR/Cas9 in intestinal stem cells, in a study performed in vitro using stem cells from cystic fibrosis patients.

An effective gene therapy for patients with cystic fibrosis will require the delivery of cDNA encoding the cystic fibrosis transmembrane conductance regulator protein to the nucleus of the epithelial cells lining the bronchial trees within the lungs. Furthermore, a successful outcome could only be achieved if the transgene is expressed for the rest of the life in the patient. So far, while it has been possible to deliver the transgene to the nucleus of the target cells, the optimal expression of the gene has not been more than 30–40 days in clinical trials. The problem of the limited expression is further complicated by the ability of the host's immune response in reducing the effectiveness of viral vectors (adenovirus and adeno-associated virus) when administered as repeated doses. To overcome the problems of the repeated dosing, nonviral vectors such as cationic liposomes have also been clinically tested, but they are less effective than viral vectors. Additional ligands are being used, which may facilitate endosomal escape or contain a nuclear localization signal. This may enhance gene delivery by cationic liposomes. The retroviral vectors are not suitable for cystic fibrosis because they require cell division, and the target cells in the airway have a very low replication rate.

11.4.7 Parkinson's Disease

Parkinson's disease is caused by the death of cells that synthesize and store dopamine in substantia nigra. It is a progressive neurological condition, which parallels the death of dopamine synthesizing cells in substantia nigra. Since there is no current cure for the disease, addressing the loss of dopamine or saving the neurons that stores dopamine from degeneration by gene therapy is a viable option. Multiple forms of gene therapy are being assessed to treat Parkinson's disease. These include symptomatic approaches such as the synthesis of ectopic L-dopa, the complete ectopic dopamine synthesis, the use of glutamic acid decarboxylase, or the ectopic L-dopa conversion. Disease-modifying gene therapies constitute the expression of GNDF (glial cell line-derived neurotrophic factor) or the regulation of the gene expression of Parkin gene and α-synuclein. The most common vector used for these trials is adeno-associated virus (AAV), although others have also used lentivirus. Delivering genes that code enzymes needed for the synthesis of dopamine, including tyrosine hydroxylase, GTP cyclohydrolase I, and aromatic L-amino acid decarboxylase, has been emphasized.

Alternatively, gene therapy has been used to address the symptoms of the patient. Some of the approaches include the ectopic dopamine synthesis. Another form of gene therapy consists of the synthesis of ectopic L-dopa in the striatum. For this treatment tyrosine hydroxylase and GTP cyclohydrolase I genes are transferred into the medium spiny neurons. The expectation is the conversion of L-dopa to dopamine by the endogenous aromatic L-amino acid decarboxylase.

There has been detection of some genes, which can regulate the phenotype of neuron or serve as neuroprotective agents. Since drugs cannot be repeatedly injected into the nigrostriatum area where the substantia nigra meets the striatum, gene ther-

apy is a viable option. As this would be a single treatment, and viral vectors would be able to diffuse and transduce into the striatum. The principal concept behind using gene therapy for Parkinson's disease is to develop novel cells, which can synthesize dopamine and subsequently transplant the cells to the patient.

Gene therapy can also result in fully ectopic dopamine synthesis. Aromatic L-amino acid decarboxylase can regulate the formation of dopamine from levodopa, which is missing in the patients with Parkinson's disease due to the loss of neurons in the nigrostriatum. The vector AAV2-AADC is designed to reestablish the normal levels of aromatic L-amino acid decarboxylase in the striatum resulting in enhanced synthesis of dopamine. This will alleviate levodopa-induced dyskinesia. Another form of gene therapy is ectopic L-dopa conversion, where AAV vectors are used to increase the efficacy of the L-dopa therapy by gene enzyme replacement. This vector transduces the AADC coding sequence into the MSN in the striatum, resulting in the conversion of L-dopa to dopamine. A different symptomatic approach uses glutamic acid decarboxylase (GAD) expression in the subthalamic nucleus, which is a gene enzyme replacement therapy. The purpose is to increase the efficacy of L-dopa utilizing AAV vectors. This AAV vector transduces the AADC coding sequence to the MSN in the striatum. This results in the conversion of administered L-dopa into dopamine. Glutamic acid decarboxylase-based NLX-P101 significantly reduces movement damage.

ProSavin is a tri-cistronic equine infectious anemia virus-based lentivector and codes for aromatic L-amino acid decarboxylase, GTP cyclohydrolase I, and a truncated form of tyrosine hydroxylase. It has been tested in 15 patients with Parkinson's disease and has exhibited a positive safety profile and significant long-term improvement in motor function, 6 and 12 months posttreatment in phase I/II trials. The follow-up data demonstrates continued lack of side effects and positive therapeutic response, for up to 4 years after the treatment. Although the study has a small sample of patients, the highest dose (5×) produces the best levels of dopaminergic activity. The patients in the highest dose group exhibit the greatest benefits based on the mean motor scores, a decrease in the need for oral dopaminergic drugs.

11.4.8 Hemophilia

Hemophilia is a rare blood-clotting disorder and is a result of mutations in the gene for coagulation factor IX (hemophilia B). The coagulation factor IX is a protein that causes the clotting of the blood. For the treatment of hemophilia by gene therapy, human factor IX gene is inserted into a recently modified vector adeno-associated virus serotype 8 (AAV8). In one study, the sample included 10 men between the ages of 22 and 64. A single intravenous infusion of vector adeno-associated virus serotype 8, carrying the transgene into the patients with severe hemophilia B, produced a dose-depended amelioration of the disease by increasing the quantity of the circulating factor IX. This increase in the levels of circulating factor IX were

observed within 4 months after the administration of the gene therapy. The activity increased from less than 1 % of normal to between 1 and 6 % of normal. The effectiveness of the gene therapy is observed for the whole monitoring period that is up to 4 years for some patients.

11.4.9 Vasculature

The gene therapy for vasculature involves delivery of the therapeutic gene(s) to many different types of vascular cells including myocardium, endothelium, vascular smooth muscle, and/or tissues involved in regulating the lipid levels. Many different protocols have been used for this form of gene therapy, which include ex vivo gene therapy, cell-based genetic alteration, delivering the gene to the target locally in vivo, and system transgene delivery. Ex vivo gene therapy can be used, if a therapeutic gene delivery is possible to a tissue safely and effectively. An example is the gene therapy of vein graft failure, which is done when a vein could be reached during a coronary artery bypass surgery, and this procedure has distinct advantages since there is a minimum immune response to the virus due to transduction with a limited amount of vector, and local delivery prevents systemic transgene expression.

The development of local delivery devices is essential in targeting vascular tissues for gene therapy since most of the tissues are inaccessible when vector is administered systematically. Catheters have been devised which can be used under x-ray fluoroscopy guidance for gene delivery. This provides a contrast medium for arterial wall gene transfer where only the lumen of the vessel could be seen. It is expected that this process in combination with magnetic resonance imaging will demonstrate the atherosclerotic lesions and genes interaction. The devices under consideration for local gene therapy are stents, channel balloon, microporous coated stents, nipple catheters, microspheres, double balloon, and hydrogel-coated catheters.

11.4.10 Cardiovascular Diseases

11.4.10.1 Ischemia

Gene therapy provides an alternative to amputation and heart transplant for the treatment of ischemia. The focus of gene therapy for ischemia has been on genes for angiogenic growth factor and fibroblast growth factor where the goal is to deliver the genes for these growth factors to the site of ischemia. Angiogenic growth factor is involved in the angiogenesis of endothelial cells. In clinical trials of patients with peripheral arterial disease, the intramuscular administration of angiogenic growth factor gene using adenovirus as the vector has demonstrated an increased

endothelial cell function and lowering of extremity flow reserve. In another study, the administration of this gene resulted in the improvement of angina symptoms. Gene therapy has been used to enhance angiogenesis in patients with periphery artery disease (critical limb ischemia). The focus has been delivering the genes for pro-angiogenic factors such as vascular endothelial growth factor, hepatocyte growth factor, and fibroblast growth factor. Naked plasmid DNA (pDNA) is used to encode these growth factors. The use of naked pDNA results in only limited transgene expression. It is expected that the use of other gene transfer systems, including ultrasound microbubbles and injectors without needles with additional genes, may improve transgene expression.

11.4.10.2 Atherosclerosis

Gene therapy for atherosclerosis is challenging since the disease is complex and involves the interaction of both the genetic and environmental factors. The gene transfer is also difficult to achieve in the vasculature specifically when the administration involves atherosclerotic lesions as targets. A number of diverse genes have been considered for the treatment of atherosclerosis. Some examples are genes for LDL and VLDL receptors, hepatic lipases and lipoprotein, apoB mRNA editing enzyme, apolipoprotein AI, and lecithin–cholesterol acyltransferase. LDL receptor efficiency, a determinant of atherosclerosis, is a major genetic abnormality. The transfer of LDL or VLDL receptor gene overcomes this LDL receptor efficiency. Apolipoprotein E (ApoE), which is a protein present in circulation, has pleiotropic atheroprotective properties and has drawn serious consideration for the treatment of cardiovascular disease and hypercholesterolemia.

11.4.10.3 Hypertension

Hypertension is a major risk factor for stroke, atherosclerosis, and peripheral vascular disease. The hyperactive renin–angiotensin system has been established as a contributing factor for primary hypertension. A clinical trial called PHACeT (Pulmonary Hypertension: Assessment of Cell Therapy) uses engineered stem cell-like “endothelial progenitor cells,” which are obtained from the blood of the patient. Since patients with pulmonary hypertension exhibit deficiencies in the production of nitric oxide, a DNA vector carrying a gene for endothelial nitric oxide-synthase (eNOS) has been added and is delivered to the endothelial progenitor cells. This enzyme is involved in the production of nitric oxide, which is a vasodilator, and also plays an important role in the repair and regeneration of blood vessels. These engineered cells carrying the transgene are injected into the lung.

The atrial natriuretic peptide (ANP) is involved in lowering the blood pressure by a number of mechanisms, which include relaxation of blood vessel’s smooth muscle cells, increasing the diameter of the blood vessel, and altering the effects of vaso-

constrictive agents. ANP is also involved in affecting the elimination of sodium and inhibiting sympathetic nervous system. In an animal model, the atrial natriuretic peptide gene was added by using a modified adenoviral vector that was also connected to a gene regulatory system turned on by the drug mifepristone. The results of the study suggest that the experiments were successful in returning the blood pressure to the normal levels in this group. Additional targets for lowering blood pressure by gene therapy include endothelin and kallikrein.

11.4.10.4 Thrombosis

The endothelial cell dysfunction results in clot formation, and the anticlotting genes are associated with antiplatelet activity. The tissue plasminogen activator gene is a suitable target to treat thrombosis; however, the continued expression of tissue plasminogen activator (TPA) gene is required since TPA has a short half-life. Additional anti-thrombosis gene products include antistatin, tissue factor pathway inhibitor, thrombomodulin, and hirudin. The anti-thrombotic gene therapy could be useful in multiple clinical conditions including peripheral artery angioplasty, percutaneous transluminal coronary angioplasty, intravascular stenting, and coronary artery bypass graft. Due to a delay in gene expression, it seems more appropriate if gene therapy for thrombosis is used for the prevention of chronic arterial narrowing and reocclusion.

11.4.10.5 Familial Hypercholesterolemia

Patients with familial hypercholesterolemia have an inherited disorder and possess deficient low-density lipoprotein receptors, resulting in the inability to process cholesterol correctly. These patients develop elevated levels of plasma cholesterol, which cause arteriosclerosis at an early age due to high levels of artery-clogging fat resulting in heart attacks and strokes. The disease provides an intricate gene therapy approach where the concept is to modify the liver so it is able to express LDL receptors. The procedure involves ex vivo transgene therapy in which there is a partial surgical removal of liver followed by insertion of corrected copies of the deficient gene and transplantation of the liver segment back to the patient.

On the basis of the experiments on gene delivery, only the adenovirus (among viral vectors) produces sufficient expression to produce a therapeutic response in some animal models, but a severe immune response remains a major hurdle. The other alternative has been the cationic liposome complex intravenous gene delivery, which has exhibited gene expression in vascular endothelial cells and monocytes/macrophages. Immune response is not a problem with these vectors. However, the efficiency of gene delivery is low that limits their use. Some of these gene delivery efficiency problems have been overcome by using ligand-facilitated transfer of cationic liposome DNA transfer.

Cell-based genetic modification has been used to treat familial hypercholesterolemia. This form of gene therapy involves the collection of the target cells from the patient, insertion of the desired genes, and regrafting of the modified autologous cells. Specifically, hepatocytes are harvested and transduced with retrovirus-expressing genes for low-density lipoprotein receptors and subsequently reimplanted to the patient, but the procedure has shown only a limited success in correcting the problem, and the success of statins has further diminished the enthusiasm for the procedure.

11.4.10.6 Human Immunodeficiency Virus

HIV infection has infected up to 73 million people worldwide and more than 1.2 million people in the United States. The development of new drugs and the awareness about the infection have not slowed down the infection rate of the virus and the resulting death rate worldwide. The treatment regimens used reduce viral loads and enhance life expectancy. The cost of the drugs and dependency on a strict compliance make their usefulness very limited, specifically in the developing countries where the infection is spreading at an alarming rate. Furthermore, the spread of drug-resistant strains has added to the concerns about the disease.

Gene therapy has been under serious consideration as an antiviral treatment. One approach has been the RNA-mediated inhibition of HIV, which provides a superior alternative to anti-retroviral agents that does not carry the risk of adverse side effects associated with the available reverse transcriptase and protease inhibitors. In addition to the antisense RNA therapy, RNA interference (RNAi) and ribozyme-based gene therapy are also under investigation. These treatments are responsible for post-transcriptional gene silencing or splicing of viral RNA and inhibit HIV transcripts or HIV receptors. The recognition of the cognate RNA sequence is required for these treatments to succeed, and the sequence-specific breakdown of HIV genome could be achieved by injecting the cell with short interfering RNA. The entry and proliferation of HIV into the $CD4^{+}$ cells can be prevented by targeting CD4 receptors and the gag gene by using RNAi. However, for these treatments to succeed, long-term expression of SiRNA is essential. Long-term expression of SiRNA would enable the sustained production of anti-HIV genes.

Since HIV has a very high mutation rate, the effectiveness of both siRNA and ribozyme sequence treatments has their limitations and reduces the impact of their treatment as HIV mutates and evades SiRNA due to its short length. Ribozyme recognition sequences are also small, and any mutation in its cleavage site would render resistance. Antisense RNA therapy overcomes these limitations because long antisense sequences targeting HIV force the virus to mutate at a much higher rate, to escape inhibition of replication caused by antisense RNA. A clinical trial employed a lentiviral vector VRX496, containing a long antisense sequence to HIV envelope while retaining the full LTRs of HIV and without its self-inactivation. It was anticipated that using long antisense sequence can target multiple sites of HIV,

which may limit the ability of the virus to form resistant mutants. For this trial, peripheral blood lymphocytes were obtained by apheresis, $CD4^+$ T cells were purified and transduced with the VRX496 vector and were then infused into the patient. All patients in the study tolerated a single IV infusion of gene-modified autologous CD4 T cells. The key observations from the study were that these patients with late-stage HIV infection retained modified $CD4^+$ T cells for long term with minimum immunogenicity. However, a complete safety profile of this gene vector therapy has not been established at this time, since there is a latency period of 3 years for adverse effects. A total of five patients were enrolled in this study; four of the five patients exhibited an increase in the cellular response to HIV, three patients had improved T-cell memory responses, and one subject produced a very strong antiviral response. Lastly, gene editing, which is the site-specific modification of the gene, appears to be safe. In another study with VRX496, multiple infusions of these lentiviral vector-modified autologous CD T lymphocytes were administered in 17 patients infected with HIV and receiving anti-retroviral therapy. Twelve patients were given six infusions and five patients were given three infusions. The anti-retroviral effects of the treatment were not statistically significant. However, the vector had an effect on the HIV, with transitions of A-to-G in the antisense region of the viral envelope in these HIV-infected patients. In another study, where CCR5 was made permanently dysfunctional by gene editing, the results showed a decrease in the levels of HIV DNA in most patients.

11.4.10.7 Epilepsy

Gene therapy is a potential viable alternative for the treatment of epileptic patients who do not respond well to the conventional antiepileptic drugs. With the use of transgene viral vectors, various gene targets, specifically the inhibitory and excitatory neurotransmitters, have been the focus of interest. In animal models, success has been reported when neuropeptide genes including neuropeptide Y and galanin have been transduced in specific areas of the brain. The intracerebral applications of the transgene were performed with an emphasis on preventing the seizures by using recombinant adeno-associated viral vectors, and the results exhibited reduction in seizures, delay in fully kindled seizures, and the production of neuroprotection. The concerns that need to be addressed before human clinical trials include the production of an immune response, specifically, the synthesis of antibodies to the viral vector, promoter silencing, stability of the transgene, loss of transduced cells, and measurements of therapeutic efficacy. In one experiment adeno-associated viral vector containing a lacZ marker gene has been used for direct gene transfer into human epileptogenic hippocampal tissue using brain slices. However, this technology has not been used for clinical practice, and the expression of the transgene in neurons will be critical for the success of such a protocol. Additional targets for these studies include genes for adenosine, galanin, neuropeptide Y, somatostatin, anti-NMDA, cholecystokinin, glut 1, neurotrophins, and calbindin.

11.5 Hematopoietic Stem Cells as a Target for Gene Therapy

Various inherited and acquired disorders could potentially be corrected by introduction of transgene into bone marrow stem cells. Hematopoietic stem cells are self-renewable and are able to differentiate and proliferate. They are readily accessible and can be easily administered back to the patient by autologous transplantation. This form of treatment would benefit a number of disease states including thalassemias, sickle cell disease, various lymphocyte disorders, chronic granulocytes diseases, chemotherapy-induced myelosuppression, and AIDS. Lentiviral vectors could be efficient tools for hematopoietic stem cells gene delivery since they are quiescent and consequently are difficult targets for vectors, that require dividing cells. However, gene therapy of hematopoietic stem cells is still limited by their quiescent nature, low frequency of the target cells, poor grafting ability of gene transduced stem cells, and inability of a growth advantage for genetically modified cells.

11.6 Challenges Associated with Successful Gene Therapy

The idea of gene therapy was born to treat diseases that are caused by defects in single genes; clinical trials have been conducted for diseases – ornithine transcarbamylase deficiency, hemophilia resulting from factor IX deficiency, chronic granulomatous disease, severe combined immunodeficiency, beta thalassemia, choroideremia, cystic fibrosis, and sickle cell disease with mixed results. Although no sustained clinical benefits were observed after gene therapy in patients with ornithine transcarbamylase deficiency and factor IX deficiency hemophilia, the development of a functional immune response was reported in patients who received gene therapy for severe combined immunodeficiency. The patients receiving gene therapy (MDS1-EVI1) for chronic granulomatous disease developed functional neutrophils and clonal myeloproliferation.

These clinical trials have pointed to a number of adverse effects, problems with in vivo administration of viral vectors and the severe concerns of insertional oncogenesis. Several problems associated with in vivo administration of viral vectors have been identified that include the following:

1. An immune response is produced against the viral vectors that make the subsequent administration of viral vectors less feasible, specifically when a sustained expression of the gene is a major issue.
2. The viral vector also infects nontarget cells resulting in oncogenesis or severe inflammatory responses.
3. Although germ line transduction has yet not been reported, its risks continue to be a major concern for the success of gene therapy protocols.

In all the patients developing cancer, the genetic defect was treated by inserting a therapeutic gene into a modified retrovirus. This vector was then infected into

bone marrow stem cells isolated from each patient and subsequently administered back into the patient. The transgene encoded the common ϒ chain of the interleukin-2 receptor (ϒc), which is defective in patients with SCID. Unfortunately, these vectors also activated a cancer-promoting gene, which was due to transduction of the retroviral vector close to the promoter of LM02 that is a proto-oncogene. The development of leukemia has been a major concern for the future of gene therapy both in Europe and the United States, due to reactions from regulatory authorities, but despite the risk of leukemia, gene therapy is a superior alternative to mismatched bone marrow transplantation for SCID patients. Nevertheless, all gene transfer techniques that lead to the integration of DNA into chromosome may cause mutagenesis due to inactivation of tumor suppressor genes or activation of a proto-oncogene. There has also been concern for patients with chronic granulomatous disease undergoing gene therapy to develop leukemia and myelodysplasia. Nevertheless, so far, the studies have not provided sufficient data to link gene therapy with oncogenesis in human clinical trials. Four possible ways have been recommended to decrease the risk of oncogenesis:

1. Improvement in vector design.
2. Buffer the genome from the effect of viral integration.
3. Controlling transgene integration.
4. Production of genetically modified stem cells in vitro and infusion of these non-oncogenic cells into the patient.

11.7 Conclusion

While significant progress has been made over the past several years in developing new technologies for gene therapy, the outcomes have not matched the expectations, and the "Gelsinger case" in Philadelphia continues to raise a red flag. The identification of therapeutically suitable genes, development of efficient viral and non-vector systems, and efficacy and safety of the clinical trials are essential to attain the high expectations from this mode of treatment.

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Chapter 12
Immunopharmacologic Approaches to Treat Cancer

Abstract Cancer is an extremely diverse disease and difficult to treat because it is not only the result of mutations in the genes that modulate cellular proliferation and growth, but it also evades the immune response. Although the survival rates have gradually improved with the understanding of the underlying mechanisms as well as the development of new drugs that target these pathways, many forms of the cancers are still incurable, depending on the stage of the diagnosis. Due to the better understanding of the disease process aided by the advances in new techniques, the emphasis for the treatment of cancer has shifted to personalized therapy by employing the modified immune system of the patient as opposed to administering indiscriminate cytotoxic agents. The use of monoclonal antibodies and their chemotherapeutic or radiation conjugates as specific immune-based treatment for cancer has already been described in Chap. 5. In this chapter, cancer vaccines, cellular immunotherapy, and antagonism of immune checkpoints are described. Chimeric antigen receptor (CAR) T-cell immunotherapy is a novel approach where patient's own T cells are redirected using a synthetic receptor. These T cells possess specificity of a monoclonal antibody as a result of using a viral vector to transfer the coding sequence. The clinical uses of CAR T cells are a major focus of this chapter, in addition to the use of cell-based therapies, which include dendritic cells, antigen-specific T cells, tumor-infiltrating lymphocytes, natural killer cells, lymphokine-activated killer (LAK) cells, and macrophages.

Keywords Cancer vaccines • Immunotherapy • Sipuleucel-T • Autologous cell-based immunotherapy • Prostate cancer • PAP-GM-CSF • Leukapheresis • CD54 • ICAM-1 • Activated dendritic cells • TLR • Type I interferons • IL-12 • Dendritic cell therapy • Immune checkpoints • PD-1 • PD-L1 • CTLA-4 • LAG3 • BTLA • VISTA • TIM3 • Adoptive T-cell transfer • TGF-β • TH17 cells • T stem memory cells • Autologous tumor-infiltrating lymphocytes • Chimeric antigen receptors • MART-1 • MAGE-A3 • NY-ESO-1 • gp100 • MARTY-1/Melan-1 • Tyrosinase • Surviving • Malignant melanoma • Anti-4-1BB • Anti-PD-1 • Anti-PD-L1 • Donor lymphocyte infusion • IL-2 • IL-7 • IL-15 • Chimeric antigen receptor (CAR) T-cell therapy • scFv • CD3-*z* • 14g2a- *z* OX40 • CD28 • ITAMS • Leukemia • Lymphoma • Non-Hodgkin's lymphoma • Diffuse large B-cell lymphoma • Acute lymphoblastic leukemia • Chronic lymphocytic leukemia • Ovarian cancer • Neuroblastoma • CD19 • CD19-BBz • CD19-28z • HER2 • Mesothelin • Cytokine-release syndrome •

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B-cell aplasia • Tumor lysis syndrome • Anti-IL-6R • Checkpoint blockade • Bruton's kinase inhibitors • BRAF inhibitors • Bcl-2 inhibitors • Allogeneic hematopoietic cell transplantation • CM-CS1 • NKG2D • TCR-inhibiting molecules • Tumor-infiltrating lymphocytes • Adoptive T-cell transfer

12.1 Introduction

Cancer is known to mankind going back to 2500 B.C. However, it was not until the sixteenth or seventeenth century that some theories regarding the reasons behind the disease were put forward. They included milk clot for breast cancer, acidic lymph fluids, and a slowly spreading poison. The evidence of metastasis was formulated in the nineteenth century that the cancer spreads from the primary tumor via the lymph nodes to other organs. During the early twentieth century, it was determined that cancer has genetic basis. Later, the development of transplantable tumors in mice resulted in the discovery that protective immune response against tumors can be produced. However, it has been questioned for a long time whether the immune system is capable of recognizing and eliminating cancer cells, whereas the development of cancer was attributed to the failure of the immune response. One mechanism that is comprised of four phases of elimination of tumor is immunoediting and is suggested to be a protective mechanism against the development of tumors. During the first phase, the antitumor response is produced by innate immunity, and there is an induction of the inflammatory response, which results in the recruitment of natural killer cells, dendritic cells, macrophages, and natural killer T cells to the site of the tumor. There is also production of IFN-ϒ during this phase. IFN-ϒ then causes the killing of the tumor and the production of certain chemokines, which constitute the elimination of phase two. This is followed by phase three in which there is further killing of tumor cells by apoptosis and free radical oxygen, after the activation of macrophages and natural killer cells, as well as the production of IL-12 and IFN-ϒ. During the last phase, there is homing of tumor antigen-specific helper and cytolytic T cells, and the destruction of the tumor by $CD8^+$ T cells. In patients with cancer, there is an escape from the elimination phases as the tumor cell variants mutate, resulting in uncontrolled proliferation and the development of the malignancy. However, the role of immune response in preventing the development of cancer has not been completely elucidated and is being investigated. Since infection causes some tumor regression, it is believed that immune response plays a role in inhibiting the proliferation of tumor cells. Recent advances have made it clear that the immune system has inherent capability to recognize and eliminate tumor cells. Based on these findings, modern treatments of cancer utilize several approaches that include cancer vaccines, adoptive T-cell therapy, and immune checkpoint blockade.

12.2 Cancer Vaccines

The principle of cancer vaccine is based on the assumption that it will activate the immune response of the patient against the tumor resulting in its complete destruction. These vaccines are comprised of tumor antigens and other immunostimulatory agents that are administered to the patient to induce tumor (antigen)-specific helper T cells, cytolytic T cells, and B cells. B cells will bind to the antigens on the tumor cells and will destroy the tumor cells either by phagocytosis or antibody-dependent cell-mediated cytotoxicity. Cytolytic T cells will destroy the tumor cells in context of MHC molecules and will also release cytokines and cytotoxic agents. Helper T cells via release of cytokines will cause the proliferation as well as augmentation of the effector function of both B cells and cytolytic T cells. One such example is preventive vaccination against human papillomavirus (HPV). It only induces antibody formation and protects against the virus and the virus-induced cervical cancer. Since most cancers are not caused by viruses, the recent emphasis has been the development of vaccines that would induce a cytolytic T-cell response. Nonetheless, there is antibody production as well, resulting from administration of these vaccines.

12.2.1 Sipuleucel-T (Provenge)

Sipuleucel-T is a patient-specific autologous cell-based treatment for asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer. This stage of cancer is also referred to as metastatic castrate-resistant (mCPRC) and androgen-independent prostate cancer (AIPC). It includes lymph node involvement and distal tumors and is designated as T4, N1, and M1c. The treatment is designed to produce an immune response against a marker that is expressed on most prostate cancers. It is composed of using patient's peripheral blood mononuclear cells and antigen-presenting cells, which are cultured with a recombinant human protein called PAP-GM-CSF. This protein includes an antigen expressed in prostate cancer tissue, PAP (prostatic acid phosphatase) that is attached to the cytokine, granulocyte–macrophage colony-stimulating factor (GM-CSF), which serves to activate the immune response, and is also involved in the maturation of antigen-presenting cells. The patient's peripheral mononuclear cells are isolated 3 days before the infusion date for the therapy. Autologous antigen-presenting cells and PAP-GM-CSF are the pharmacophores of Sipuleucel-T. During tissue cultures, the recombinant antigen binds, taken up, and processed by the antigen-presenting cells, where it is shredded into small pieces. The purpose is the generation of immune response directed against the antigen (PAP) by using antigen-presenting cells. Sipuleucel-T has negligible detectable levels of PAP-GM-CSF.

The composition of Sipuleucel-T is dependent on the types of cells isolated from patient's leukapheresis. Cell types in the final drug include T cells, B cells, natural killer cells, antigen-presenting cells (specifically dendritic cells), and other cells.

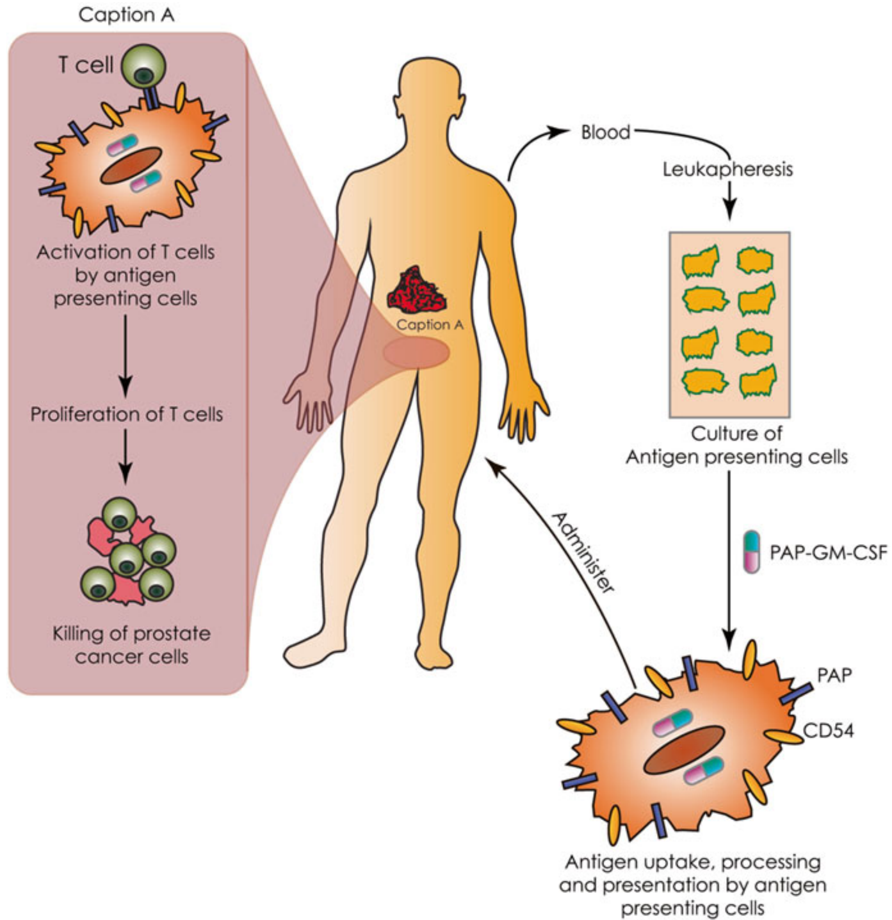

Fig. 12.1 A prostate cancer patient's own dendritic cells are isolated and are incubated with a fusion protein (PA2024). The procedure is conducted in two steps. First, they are treated with prostatic acid phosphatase, a marker that is expressed on 95 % of prostate cancer cells. This is followed by incubation with granulocyte–macrophage colony-stimulating factor. The second step allows the maturation of antigen-presenting cells. The activated cells are infused back to the patient to produce an immune response directed against prostate cancer cells expressing PAP marker. This autologous cell treatment is given three times with 2 weeks intervals

The number and composition of these cells would vary from patient to patient. However, each dose of Sipuleucel-T has a minimum of 50 million autologous $CD54^{+}$ cells activated with PAP-GM-CSF per 250 mL. CD54 also known as ICAM-1 (intercellular adhesion molecule 1) is an adhesion molecule, which binds to integrin and is involved in interaction between the antigen-presenting cells and T cells, and is identified as immune cell activation marker. The expression of CD54 in a preparation of Sipuleucel-T also reflects its potency.

Each cellular immunotherapy infusion is comprised of the maximum number of cells, which are generated by tissue culture, specific for each patient from a single leukapheresis procedure. The number of the cells in Sipuleucel-T is the same as the number of cells isolated from a single leukapheresis. Activated blood product (PAP, GM-CSF, and patient's own peripheral blood cell) is infused back into the patient to generate an immune response against cells expressing PAP antigen (Fig. 12.1). The recommended immunotherapy with Sipuleucel-T 3 is composed of a minimum of 50 million CD54+ autologous cells, infused at approximately 2 weeks interval. It is administered intravenously over a 60 min period. The patient is given acetaminophen and an antihistamine orally, 30 min before the administration to reduce the infusion reactions which include chills and/or fever.

The side effects of Sipuleucel-T include chills, headache, fever, nausea, back pain, joint ache, and fatigue. These side effects are observed within a first few days of treatment. More serious side effects are cerebrovascular events, which are exhibited in approximately 2.4 % of the treated patients as opposed to 1.8 % of control subjects.

Administration of chemotherapy or the use of immunosuppressive agents in combination with Sipuleucel-T immunotherapy has not been studied. Since this cellular immunotherapy induces the immune response, giving immunosuppressive agent(s) may delay its intended effect and even produce adverse effects. As a consequence, discontinuation of the immunosuppressive regimen or its reduction is clinically preferable.

In the IMPACT trial that served as the basis of its approval by the FDA, the median survival time was 25.8 months in patients treated with Sipuleucel-T as opposed to 21.7 months in placebo control. A different clinical trial resulted in the survival rate of 25.9 months in patients treated with Sipuleucel-T compared with placebo, which was 21.4 month. The differences in prior two studies were statistically significantly different. In a third trial, the survival rate was 19.0 months in patients treated with Sipuleucel-T as opposed to a placebo rate of 15.3 months, which was not statistically significantly different. However, the sample size was small in the third study.

12.2.2 Other Cancer Vaccines Undergoing Trials

To treat vulvar intraepithelial neoplasia, a peptide-based vaccine obtained from HPV16A cured 9 of 19 patients. A gp100 peptide vaccine in combination with IL-2 has been studied in phase III trial to treat melanoma. When compared with IL-2 alone, the survival rate was higher in this study. In another study, a personalized B-cell idiotype protein vaccine was used to treat follicular B-cell lymphoma patients who were in remission after chemotherapy. The results of this clinical trial demonstrated the disease-free survival for these patients increased from 31 to 44 months. These three vaccines are made up of known tumor antigens that were treated to increase their ability to induce immune response before administration, HPV

peptides, or gp100 in an adjuvant, and GM-CSF is mixed with the purified idiotype protein. An alternative approach is to modify the microenvironment of the tumor instead of focusing on the antigens of the cancer cells. This modification will result in an immune response by the patient against the tumor antigens. It was achieved in patients with B-cell and T-cell lymphoma where external beam radiation was combined with intratumoral administration of CpG, which is an antigen-presenting cell-activating Toll-like receptor 9 (TLR9) agonist. The results showed that there was regression in injected as well as distant un-injected tumor masses. This was attributed to the tumor antigen release after irradiation and propagation of an antitumor immune response based on changes in the microenvironment of the tumor caused by CpG.

12.3 Endogenous Immunostimulating Agents to Treat Cancer

The most potent endogenous cells to induce and prime de novo T cells are the activated dendritic cells. Toll-like receptors when bound to their respective ligand activate dendritic cells. This results in enhanced expression of various cytokines, including type I interferons and IL-12, and a number of costimulatory molecules, CD40, CD80, and CD86. These cytokines and molecules are crucial in activating T cells. Superficial basal-cell carcinoma responded to a TLR7 agonist (imiquimod) by exhibiting 80–90 % clearance in clinical trials. However, it was not efficacious in advanced cancer. Furthermore, TLR9 agonists have been used to treat melanoma and renal carcinoma, which resulted in the upregulation of interferon-α and other molecules associated with induction of immune response. These agonists have provided marginal regression of the disease, and the desired outcome was not achieved. The results obtained with other TLR agonists have also not been very encouraging in treating various forms of cancer. Nonetheless, TLR ligands activate dendritic cells and enhance T-cell priming and therefore are effective adjuvants in combination with other cancer peptide vaccines. Both imiquimod and CpG enhanced the effects of antigen-specific peptides used for vaccination to treat melanoma. In addition, dendritic cell-based vaccines that directed against glioblastoma-specific antigens have effectively utilized TLR ligands.

CD-40-specific antibodies have been used to activate dendritic cell-based vaccines, which were pulsed with tumor cell lysates or some specific tumor antigen to treat melanoma, colorectal cancer, and multiple myeloma. Antigen-specific T-cell response was observed in most patients in these trials, and the outcomes were variable. A technique has been the used that involves electroporation to add mRNA encoding CD40 ligand, constitutively active CD70, TLR4, and several melanoma antigens into autologous dendritic cells. The vaccine was administered to the melanoma patients, and 6 out of 17 patients exhibited shrinkage in their tumors. These patients also received interferon-α-2b. Furthermore, in a majority of the patients receiving this melanoma vaccine (TriMix-DC), there was antigen-specific T-cell

infiltration into the skin sites of hypersensitivity reaction. Based on these observations, the value of CD40 manipulations could be of value in treating cancer.

12.3.1 Dendritic Cell Therapy

Dendritic cell therapy is designed to cause antitumor response by inducing dendritic cells to present tumor antigens to T cells. The dendritic cells present antigens to lymphocytes, resulting in the activation and priming of the lymphocytes, eventually leading to the killing of the antigen by the lymphocyte. They are used principally for presenting cancer antigens. Vaccination with short peptides (that corresponds with antigens on tumor cell) causes dendritic cells to present tumor antigens. These peptides can be administered with an adjuvant, since they may not be potent inducers of the immune response only by themselves. In addition, cytokines are also used in combination with the antigen. For example, granulocyte–macrophage colony-stimulating factor activates dendritic cells. Alternatively, dendritic cells can be activated in vivo by genetically engineering tumor cells, which secrete GM-CSF. Ex vivo induction of dendritic cells can also be performed after isolating the dendritic cells of a cancer patient. This is normally done in the presence of a single tumor-specific peptide or a tumor cell lysate. Antibodies which bind to dendritic cells have also been used. The targets for antibodies include various dendritic cell receptors including CD40, TLR3, TLR7, and TLR8. These antibodies are modified by the addition of tumor antigens. This causes the dendritic cells to mature and produce an immune response against the tumor cells. Dendritic cells also play a role in adoptive T-cell immunotherapy. Induction and activation of dendritic cells to present tumor antigens can produce cancer-specific immune response. The variability of the number of dendritic cells generated from patient to patient is a challenge and could result in an ineffective T-cell immune response.

12.3.2 Adoptive T-Cell Transfer

A variety of cellular therapies have been tested to treat cancer. There are also relatively simple protocols to propagate therapeutic immune cells on a large scale. Some examples include the infusion of antigen-specific T cells, natural killer cells, lymphokine-activated killer (LAK) cells, macrophages, and dendritic cells. Many previous forms of adoptive cellular therapies to treat cancer were unsuccessful. Initial observations lead to the use of clinical allogeneic bone marrow transplantation for cancer treatment since the graft itself had antileukemia properties. A critical finding for adoptive T-cell therapy was that preventing the relapse of leukemia was more feasible when siblings were used as donors of the hematopoietic stem cells, as opposed to syngeneic donors for transplantation. The original principles of T-cell transfer form the basis of modern adoptive T-cell immunotherapy. In a lymphopenic

host, adoptively transferred T cells engraft and proliferate well depending on the presence of T-cell-specific cytokines and the environment of the host. An optimally engineered T cell would depend on the type of cancer and the goals of the immunotherapy. In this context, the understanding is that as opposed to effector T cells, infusion of naïve T cells, TH17 cells, and T stem memory cells may be preferable due to their ability to proliferate rapidly, for adoptive therapy. However, all of the choices have drawbacks and limitations. The use of a combination of T-cell subsets that are engineered to avoid negative feedback from TGF-β and regulatory T cells, for adoptive T-cell transfer, perhaps is preferable.

Immunotherapy using T cells is an emerging new treatment for patients with unresectable stage III and stage IV metastatic melanoma. This involves the adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) in combination with interleukin-2. The current T-cell therapies under trials to treat advanced melanoma include melanoma tumor antigen-specific T cells (HLA-binding MART-1 peptides), addition of high-affinity TCRs that detect tumor antigens to T cells, and transfer of chimeric antigen receptors (CARs). CARs are hybrid immunoglobulin light chains with endodomains of molecules responsible for the signaling of T cells that recognize specific tumor cells. The trials performed using TILs and IL-2 have produced an acceptable response in 50 % of the patients who did not do well following other treatments. In cases where TILs are not possible, T-cell therapy has been used by transducing high-affinity TCRs, specific for melanoma tumor cell antigens into the T cells obtained by isolation of the peripheral blood lymphocytes of the patient. These melanoma tumor antigens include MART-1, MAGE-A3, and NY-ESO-1.

For the treatment of melanoma, TIL therapy has been relatively successful as compared to other treatments for lasting remission. In one study, 20 % of the patients from a total of 93 patients were in complete remission for more than 3 years. This therapy allows a wider recognition by T cells of many different types of HLA-restricted tumor antigens. Another advantage of this treatment is the observation that cytolytic T cells obtained from TILs are not able to recognize over-expressed self/melanocyte differentiation antigens. These antigens include gp100, MARTY-1/Melan-1, tyrosinase, and survivin. They recognize other unknown antigens. However, there are limitations for this procedure and as a result it has not yet been included in the standard melanoma care. Selections of suitable patients for the treatment remain an issue, since there are a few predictive markers. There have been some advances in identifying the subset of TILs with desired activity, and they point to cytolytic T cells ($CD8^+$ T cells). But the specific subset with pharmacologic activity has not yet been identified. The propagation of TILs in cultures also requires improvements, and specific biomarkers will be needed to isolate the desired subset of $CD8^+$ T cells. It has been observed that patients previously treated with cytokines such as IL-2 and/or ipilimumab exhibit better response to TIL immunotherapy which indicates synergy in these treatments resulting in better outcome. The main barriers to adoptive T-cell therapy include difficulty in culturing and generating

tumor-infiltrating lymphocytes, the need for MHC presentation of antigens, and immune tolerance to self-antigens.

Allogeneic bone marrow transplantation is used in a number of blood cell-related cancers. It was observed that T cells and natural killer cells were involved in attacking the leukemia after transplantation. Combining natural killer cells with T cells has a value because NK cells do not cause graft-versus-host disease and may also contribute to inhibition of a lack of response to T-cell therapy, via development of MHC class I-deficient cancer cells. Chronic myeloid leukemia treated with transplantation seems to respond better to the manipulation of immune response including donor lymphocyte infusion (DLI). The most common treatment for chronic myeloid leukemia is the tyrosine kinase inhibitors including imatinib, nilotinib, and dasatinib. However, despite being very expensive, they are not curative but are capable of a long-term remission. As a consequence, a combination of tyrosine kinase inhibitors and adoptive cell therapy may provide a lasting cure.

For treating cancer by cell immunotherapy, preconditioning of the patient is important before the administration of the therapy. The host conditioning is done by using cytotoxic agents such as cyclophosphamide, total body irradiation, or a combination of both. This procedure results in a reduction of the tumor burden that improves the effector to target ratio. It also decreases the number of regulatory T cells and causes the production of cytokines, which augments the production of transferred T cells.

For T-cell transfer, infusion of small number of cells is enough, since there is a proliferation of T cells in the host. This negates the need for in vivo proliferation of T cells. A number of techniques have been developed for T-cell engineering that employ viruses for transduction and long-term gene expression. These transgenes have low immunogenicity. Furthermore, in some cases, permanent transgene expression may not be needed for the required therapeutic effect. Using in vitro-transcribed mRNA electroporation of lymphocytes results in temporary expression of proteins. The protocols using dendritic cells, which are transduced with mRNA, have been clinically used in addition to mRNA-electroporated T cells and natural killer cells.

Some modification of T cells is required to target tumors because tolerance to most tumor antigens in addition to an immunosuppressive microenvironment around tumor makes it difficult for a native antitumor T cell to be successful as an immunotherapeutic agent. Several adoptive T-cell therapy approaches have been employed to treat tumors. In a subset of patients with advanced metastatic melanoma, the adoptive transfer of tumor-infiltrating T lymphocytes that isolated from resected melanoma samples and propagated in vitro has been infused. In some patients, the results were durable tumor regression. There is a great promise for this technique to deliver patient-specific immunotherapy. However, there are also significant safety concerns. There is a potential for the development of graft-versus-host-like disease, if the healthy tissue expresses the same antigens as the tumor.

12.3.3 Chimeric Antigen Receptor (CAR) T-Cell Immunotherapy

Adoptive transfer of T cells expressing chimeric antigen receptors is a novel emerging paradigm for the treatment of cancer. Chimeric antigen receptors are synthetic molecules that are composed of an antigen-binding region, a transmembrane domain, and one or more intracellular T-cell signaling domain. The antigen-binding region is generally obtained from an antibody, and the transmembrane domain binds the CAR to T cells. Since T cells recognize CAR without the context of MHC molecules, this would not develop anergy. This form of immunotherapy is under the development for a variety of cancers. The main requirement is that CAR is recognized by a protein molecule on the cell surface of tumor cells. Various techniques have been used to insert CAR into the T cells. Chimeric antigen receptor (CAR) T-cell therapy is a form of immunotherapy where patient's own T cells are redirected with a synthetic receptor prepared by using a technique under ex vivo conditions. The specificity of a monoclonal antibody is introduced onto a T cell by using a viral vector to transfer the coding sequence. They include using integrating vectors, which allows a permanent integration and expression producing continuous function. Other form of integration is transient without CAR integration. The retroviruses and lentiviruses are examples of integrating vectors, whereas adenoviruses, RNA electroporation, or plasmids are used for non-integration of CAR. The CAR expression is a variable depending on the technique and vector employed, and the toxicity and the continued expression of the receptor are important issues. The survival, proliferation, and effector function of T cells are modulated by the intracellular domains included in the CAR.

This modification is for the recognition of a specific tumor antigen, and the T cells are then administered back into the patient. The technique is based on the premise that CAR-modified T cells will target any tumor-associated receptors. The administration of gene-modified T cells, specific for a tumor antigen, provides long-term disease control similar to adoptive T-cell therapy, with a rapid onset of action observed with cytotoxic agents or monoclonal antibodies. Furthermore, modification of T cells to exhibit chimeric antigen receptor prepared with the antibody fragments overcomes MHC restrictions and the challenges of immune tolerance.

Commonly, these molecules are prepared after fusion of single-chain variable fragments (scFv), isolated from monoclonal antibodies, with CD3-*z* transmembrane and endodomain. These molecules transmit *z* signal when scFv recognizes its target. scFv is formed by using a flexible linker to fuse the variable regions of immunoglobulin light and heavy chains. A single peptide before scFv causes the surface expression of the nascent protein in the endoplasmic reticulum and is also responsible for its migration to the endoplasmic reticulum. One example of this product is 14g2a- *z* which is a result of scFv fusion. scFv is obtained from hybridoma 14g2a, which recognizes disialoganglioside GD2. T cells expressing this molecule will identify and attack the cells expressing GD2, which is expressed on neuroblastoma cells. The movement of scFv in different directions is rendered by the flexible spacer

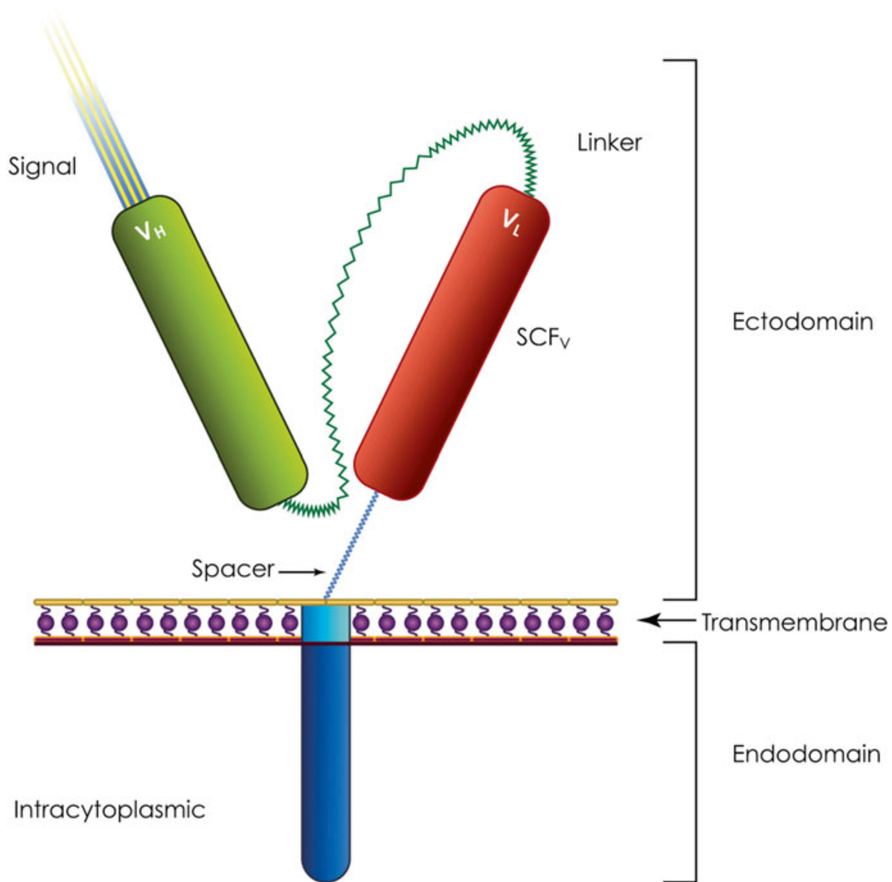

Fig. 12.2 Various parts of an artificial T-cell receptor. The recognition of antigen on tumor cell by scFv (Adopted from Mxpule)

culminating in binding to the antigen. The transmembrane domain is composed of a hydrophobic α-helix, which is obtained from signaling endodomain. This is responsible for the transmission of the desired signal as it extends into the cell (Fig. 12.2).

scFv is generally the antigen recognition domain. However, there are other molecules which serve this purpose as well. For example, native T-cell receptor α and T-cell receptor β single chains, CD4 ectodomain, and linked cytokines may possess antigen recognition domains. The antigen-binding domain is linked to a transmembrane domain by a spacer region. The most important requirement is its flexibility that will allow antigen recognition by the antigen-binding domain. CD3-*z* that contains three ITAMS is the most commonly used endodomain component. A signal is transmitted to the cell after antigen recognition and clustering of the receptors. CD3-*z* provides an activation signal after antigen is recognized by the T-cell receptor, but costimulatory signals, CD28, and OX40 are also required.

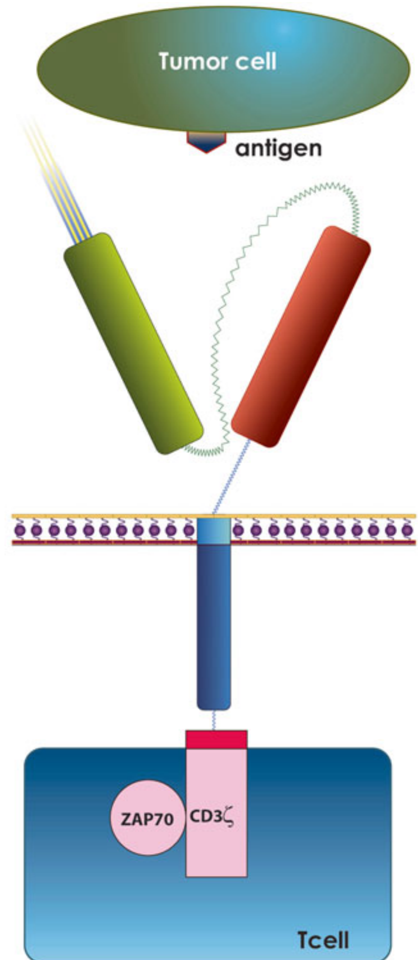

Fig. 12.3 Illustration of first-generation chimeric receptors. The recognition of antigen on tumor cell by scFv and downstream T-cell signal transduction molecules (Adopted from Casucci M, Bondanza A, www.jcancer.org/v02p0378, public domain)

The first-generation CARs possess the intracellular domain from the CD3-*z* polypeptide chain (Fig. 12.3), whereas the second-generation CARs, in addition, also include intracellular signaling domains from costimulatory protein receptors (Fig. 12.4). These costimulatory protein receptors include CD28 and ICOS and were added to the cytoplasmic tail of the CAR. The second-generation CARS are more effective than the first-generation CARS in their immunopharmacologic effect on tumor cells, as these additions rendered enhanced signaling to the T cells. The third-generation CARs (Fig. 12.5) include intracellular signaling domains from costimulatory proteins such as CD27, OX40 (CD134), and 4-1BB (CD137), which are members of the tumor necrosis factor receptor family, and their inclusion into CD3 requires CD3*z* transmembrane domain. The costimulatory domains are added to maximize the activation of T cells. The hinge and transmembrane domains of CD8-α or CD28 are generally used. These are important for interaction with the antigen, formation of the immunologic synapse, and association of CAR with other transduc-

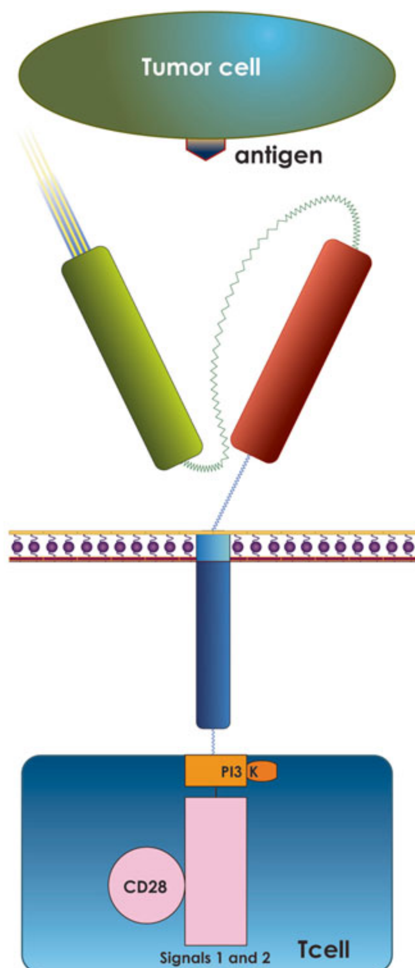

Fig. 12.4 Depiction of second-generation chimeric receptors. The recognition of antigen on tumor cell by scFv. The second-generation CAR includes signaling molecules such as CD28, 41BB, and ICOS, which are costimulatory protein receptors (Adopted from Casucci M, Bondanza A, www.jcancer.org/v02p0378, public domain)

tion proteins. Furthermore, hinge domains derived from Fc regions are under investigation, where their length is modified, and there is engagement of Fc receptors as well as activation of innate immune response. The homodimerization of CARS is separate from the TCR. It has been proposed that the affinity between the target and the CAR T cells is not as important as the spacing and conformation of binding to the epitope. CAR-directed T cells are very sensitive to low levels of cognate antigen. These cells can signal about 50 molecules for one target as compared to native T cells, which target one to ten molecules for the induction of TCR. As a result, there are multiple ways of downstream signal transduction pathways.

A variety of methods have been used to add the CAR constructs into T cells. The original approach involved the use of nonviral-based transfection, because of their cost-effectiveness and the inability to be mutagenic. However, because of long-term cell culture, the need for antibiotics inhibited the activity of the transformed cells and also caused them to become immunogenic. The transposon-based systems are

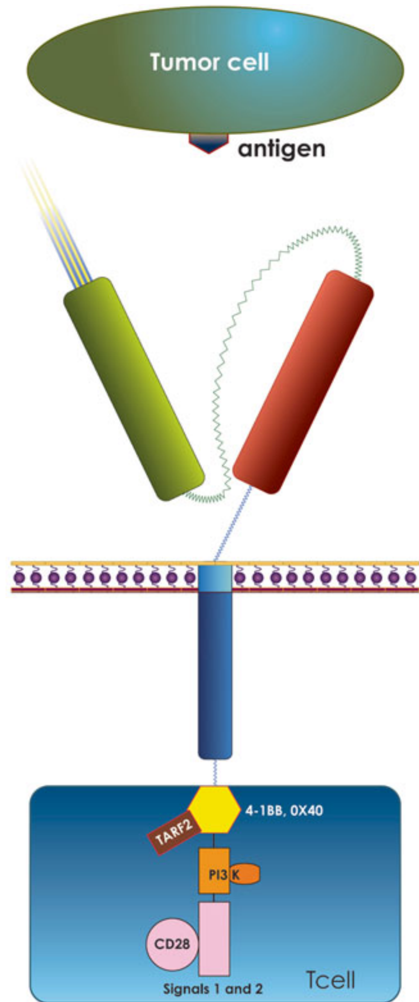

Fig. 12.5 Depiction of third-generation chimeric receptors. The recognition of antigen on tumor cell by scFv. The third-generation CAR includes signaling molecules such as CD3z-CD28-41BB or CD3z-CD28-OX40. The ultimate result is an increase in the potency (Adopted from Casucci M, Bondanza A, www.jcancer.org/v02p0378, public domain)

more efficient for the integration of the transgene. A stable gene transfer is obtained by a system called Sleeping Beauty (SB) in T cells and is undergoing clinical trials. The most commonly employed technique is the use of gamma retroviruses. They are easily propagated; the transduction is permanent and has no major safety issues in the preliminary trials, when added to the T cells. However, retroviruses have some disadvantages including the chance of silencing the expression of CAR. Alternatively, lentiviral vectors are also safe, efficient, permanent transducers but come with higher cost. Lentiviral transduction along with specific promoters allows long-term expression of CARs on T cells which would be advantageous therapeutically. The role of cytokines including IL-2, IL-7, and IL-15 has been explored in providing support for CAR T-cell immunotherapy. IL-7 and IL-15 are expected to switch off the inhibition of regulatory T cells, thus supporting the adoptively transferred T cells (Fig. 12.6).

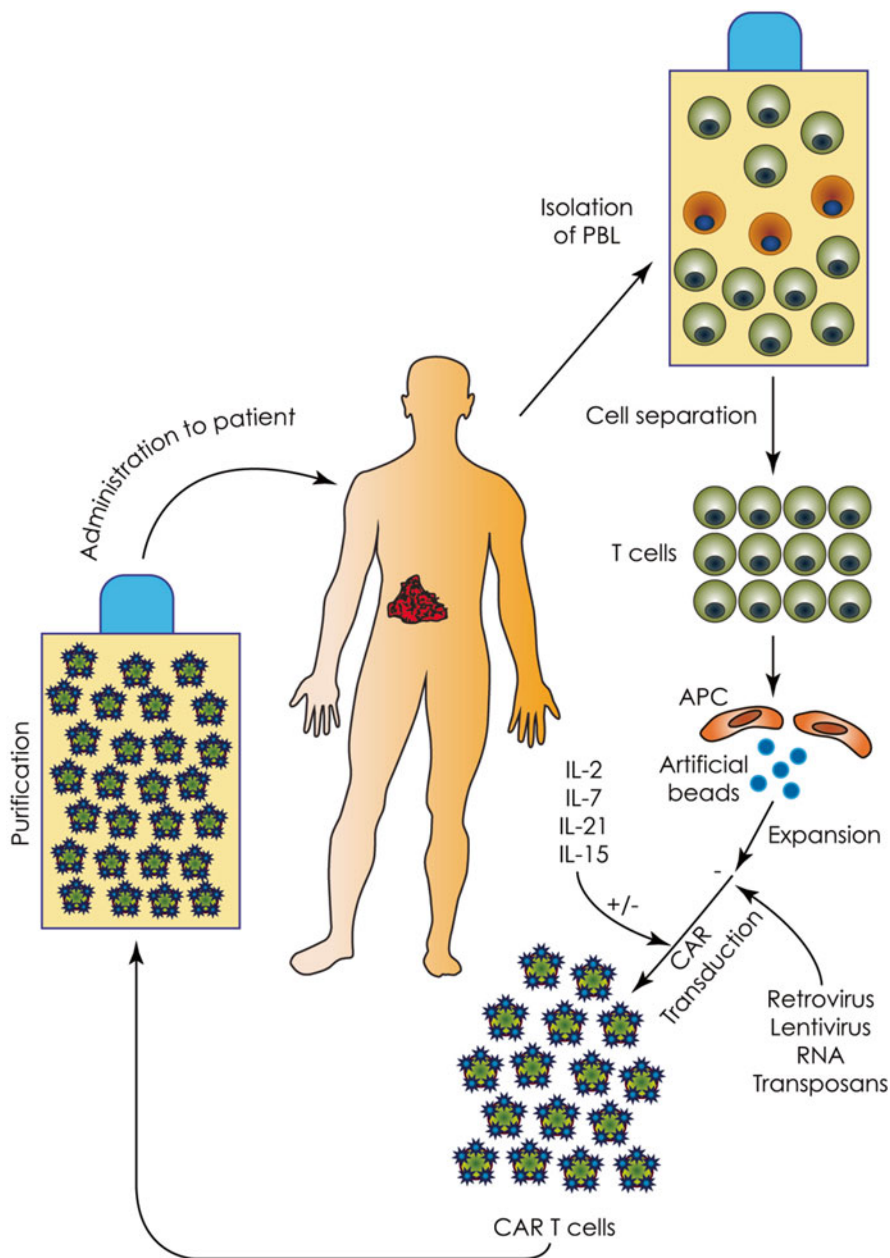

Fig. 12.6 CAR T-cell treatment involves using patient's own engineered T cells to treat cancer. In this technique, T cells are isolated from the patient's blood and are engineered genetically to express specific receptors on their cell surface that are called chimeric antigen receptors. The engineered cells are propagated through tissue culture and are then infused into the patient. These T cells recognize specific antigens on tumor cells. The expectation is that CAR T cells will proliferate in the patient's body. They will reach their target cancer cells, recognizing and killing them

12.3.3.1 Clinical Use of CAR T Cells

CAR T cells have been initially tested in blood cancers because of the familiarity of the antigens on these cells, the simple procedure for sampling tumors, and the advantage of T-cell homing. The clinical application of chimeric antigen receptors is dependent on altering the T-cell receptor in such a way that it becomes specific for a tumor cell resulting in its killing. The first-generation of CAR-modified T cells are undergoing clinical trials to treat various types of leukemias, lymphomas, including non-Hodgkin's lymphoma (diffuse large B-cell lymphoma), acute lymphoblastic leukemia, chronic lymphocytic leukemia, ovarian cancer, and neuroblastoma.

The protocol was initially used on three patients who were administered a single dose of CAR T cells directed against CD19 to treat chronic lymphocytic leukemia (CLL). CD19 is expressed on B cells from earliest B-lineage cells during the development of B-cell blasts but is not expressed on plasma cells. As a result, the antibody-secreting plasma B cells will not be affected. The treatment resulted in a long-lasting remission in these patients. Most of these studies employed a CD28 intracellular signaling domain (CD19-28z), while others used a 4-1BB intracellular domain (CD19-BBz) along with CAR. In initial studies, the presence of CAR T cells was consistent in the blood, bone marrow, and spinal fluids of the patients. Furthermore, the persistence of CAR T-cell levels correlated with the response to treatment.

A serious side effect in patients following CAR T-cell treatment was found in a patient who has colorectal cancer metastases to the lung and the liver. This patient was treated with a third-generation CAR specific for epidermal growth factor receptor 2. The CAR contained scFv obtained from Herceptin. There was respiratory distress within 15 min after treatment, which was followed by multiple cardiac arrests resulting in death. Based on the serum analysis performed 4 h after administration of CAR T cells, it was noted that CAR T cells were not present in tumor metastasis, and there was significant increase in a number of cytokines including IL-6, IL-10, GM-CSF, TNF-α, and IFN-ϒ. The multi-organ failure was attributed to the recognition of HER2 in lung epithelium that caused the production of inflammatory cytokines and pulmonary toxicity. A different antibody is being used to develop second-generation HER2-targerted CARs and clinical trials that are ongoing, using a lower dose for a number of HER2-overexpressing malignancies.

Mesothelin is an antigen that is overexpressed in pancreatic, ovarian, malignant pleural mesotheliomas, and a subset of lung cancers. But its expression is low in normal tissues. Targeting mesothelin second-generation CARs was evaluated for their safety, where mRNA electroporation (CARTmeso) was utilized for CAR expression. One patient tolerated this treatment well, while the other patient went into cardiac arrest due to anaphylactic shock. The anaphylactic shock was attributed to human anti-mouse antibodies specific for the mouse scFv in the mesothelin CAR. Lentivirus-engineered CART meso cells are planned in a phase I study after repeated administration of CAR T cells with mouse components that was found to be safe in 20 out of 21 doses in four patients.

The patients with B-cell malignancies have shown success when treated with CAR T-cell immunotherapy. A second-generation CAR T cells, derived from mouse antibody specific for human CD19 and a CD28 costimulatory signaling domain, were used to treat a patient with follicular lymphoma. The partial remission up to 32 weeks was noted, and there were no treatment-related acute side effects associated with this treatment. After 9–39 weeks of treatment, there was a decrease in the immunoglobulin, IgG, IGM, and IgA levels in the serum. The trial eventually produced remission in five out of seven patients with one patient achieving complete remission. There was depletion of B cells and decrease in serum antibody levels in four out of the eight patients. In addition, these CAR T cells have been used to successfully treat diffuse large B-cell lymphoma. These patients also experienced some neurological toxicities. In another study, there was 70 % complete remission when these CAR T cells were used in 21 patients with B-precursor acute lymphoblastic leukemia. Furthermore, anti-IL-6 R (CD 19)-targeted second-generation CARS have been developed using different antibodies and encoding various second costimulatory molecules and are undergoing clinical trials. It seems the susceptibility to therapy differs despite using CD19 as the target antigen. The response was better among ALL than CLL patients. This has been attributed to many reasons including the defects in host's T cells in lymphoma patients, immunosuppressive microenvironment of the tumor, patient's age, history of prior treatment, and nature of the infused drug.

The development of CARs for solid tumors has been difficult due to lack of identifiable cell surface markers on malignant epithelial cells. It is also not known whether some specific markers are exclusively expressed on tumor cells or a marker is overexpressed on tumor cells, as opposed to the normal tissue. The interpretations of the results are complex because each trial center has developed its own CARs that differ in single-chain variable fragments, signaling domains, method of placing CAR gene into T cells, conditioning regimens, and post-CAR T-cell infusion interventions. The responses in acute lymphoblastic leukemia have been very encouraging, whereas this is not the case in treating chronic lymphocytic leukemia, using CD19 CAR T cells. The clinical responses are dependent on the distribution and duration of survival of CAR T cells.

12.3.3.2 Challenges Associated with CAR Therapy

The number of T cells required to be administered remains a challenge due to the complexity associated with T-cell activation, differentiation, and homeostasis. Cultured T cells irrespective of the allogeneic stimulus exhibit a differentiated phenotype accompanied by losing their ability to proliferate and engraft. Furthermore, there in vivo function is compromised. The available culture systems have many drawbacks for their use in T-cell immunotherapy. Another option is third-party donor banks, but due to the diverse MHC types in individuals, they are expensive. To overcome the issues of survival and effector function of adoptively transferred T cells, virus (EBV or CMV)-specific T cells are used and redirected against antigens

associated with tumors. Other alternatives that have been used include naïve T cells and central memory T cells, which have long life. The stem cell memory T cells are undifferentiated and have the ability for long survival without an allo-antigenic stimulus. As they possess memory cell function, this should be an advantage for long-term remission of cancer. The adoptive T-cell therapy is an effector product of cytotoxic T cells; however, natural killer cell-based therapies have been performed, and the use of isolated helper T cells has shown tumor regression.

12.3.3.3 Side Effects of CAR Therapy

The side effects of CAR immunotherapy include cytokine-release syndrome, B-cell aplasia, and tumor lysis syndrome. All of the side effects correlate with the success of CAR T-cell treatment. The expansion of CAR T cells in the patient produce cytokine-release syndrome characterized by fever, chills, nausea, hypoxia, adverse effects on the nervous system, and malaise. The neurological effects also include changes in mood, seizures, and aphasia. There is an increase in IL-6 levels, and treatment with tocilizumab (anti-IL-6R) helps with the symptoms of cytokine-release syndrome. The targeting of CD19 antigen produces B-cell aplasia. This is not a major issue since B cells are not an essential tissue for survival. The tumor lysis syndrome can be observed 1 or 2 months after the CAR T-cell therapy. Antibody deficiency can be treated with intravenous immunoglobulins. The usefulness of this treatment for a tissue essential for survival remains a challenge. Other side effects included renal failure, and hypotension in some patients, which are attributed to the high levels of TNF-α and IFN-ϒ.

There are also tissue toxicity concerns with CAR T-cell therapy, and safety-check mechanisms are required. However, the function of CAR T cells may be affected by the safety mechanisms. The toxicities could be for multiple reasons. They could be due to extrinsic factors during the tissue culture, the result of cytokines that were added to the cells, and the therapeutic cells themselves. Cytotoxic T lymphocytes administered to treat Epstein–Barr virus (EBV)-associated lymphomas produced respiratory obstruction. This side effect is attributed to T-cell-induced inflammatory response usually observed after tumor edema or necrosis. The infused T cells may cause tissue damage as observed in autoimmune diseases, which are T-cell mediated. Graft-versus-host disease and bone marrow aplasia can occur in patients not receiving autologous lymphocytes for treatment. The patients treated with CAR T-cell therapy for B-cell malignancies present cytokine-release syndrome, when the levels of CAR T cells are at a maximum level in blood and bone marrow, and thus these symptoms are delayed. The side effects are managed by cytokine antagonists or monoclonal antibodies, in addition to corticosteroid in patients with acute lymphoblastic leukemia and chronic lymphocytic leukemia. Other possible toxicities associated with this treatment may include production of viruses (retroviruses, lentiviruses) that may be able to replicate the possibility of malignancy and development of T-cell lymphoproliferative disorders. However, based on the clinical trials, the risk for developing these disorders is relatively low.

12.3.3.4 CAR T Cells in Allogeneic Stem Cell Transplantation

Allogeneic hematopoietic cell transplantation is not successful in many instances in treating leukemia, and the attempts to achieve the graft attacking leukemia, as opposed to the host, have not been realized. The relapse of leukemia is generally treated with donor's normal lymphocyte infusions, and one of the outcomes is the development of graft-versus-host disease. This treatment is only successful in patients with relapsed chronic myeloid leukemia but not in relapsed acute lymphoblastic leukemia. It has been suggested that CAR T cell therapy may be an option to improve the effects of allogeneic human stem cell transplantation or the benefits of foreign lymphocyte infusions. This possibility is under investigation in a number of clinical trials, where the safety and efficacy of CAR-modified allogeneic T cell infusions are under assessment to treat leukemia. The questions have been raised regarding the use of this immunotherapy in normal clinical practice and whether alternate potential antigenic targets, besides CD19- directed CAR T cells, can be used. Graft-versus-host disease is a problem in patients who receive donor lymphocyte infusion after relapse and have previously gone through transplantation. To remedy this situation, CAR T cell therapy can be explored for remission to replace donor lymphocytes infusions in patients with acute lymphoblastic leukemia experiencing relapse, after undergoing transplantation. Multiple vaccine strategies could also be employed, and using viral specific T cells as the transduced cell population may overcome the issue of the graft-versus-host disease.

12.3.3.5 Monitoring of Infused Cells

The questions have been raised regarding the uniform-data reporting guidelines for the systemic analysis of information obtained from the clinical trials of T-cell therapy. This information will be helpful in reference to the efficacy of these treatments and their mechanism of action. The idea is to identify benchmarks that correlate with the effectiveness of the T-cell therapy. This will suggest an endpoint for the treatment, as was the case with gene therapy or HIV vaccines; this therapy involves monitoring the state of not only the disease but also the treatment itself. Furthermore, it involves biologically viable and dividing pharmacologic agents that interact with the host in a number of circumstances. The use of adoptive transfer of bulk tumor-infiltrating cells to treat melanoma resulting in its regression, followed by using gene-MART-1-specific T cells to treat cancer, set the foundation of these studies. There was a correlation between survival and proliferation of infused cells, regression of the disease, and duration of the remission. In animal studies, a role of central memory cells in T-cell therapy has been suggested. Some naïve and memory T-cell subsets may possess phenotypic plasticity. It has not been possible to measure the activity of the infused cells directly. The indirect measurements include quantitating the cytokine levels in the infused patients. The phenotype and effector status of T cells that will produce optimal antitumor efficacy is still being debated.

The mechanisms employed by T cells to kill tumors include cytotoxicity, synthesis and secretion of cytokines, and production of chemokines, all of which work in combination to produce tumor-killing response. The survival and effector function of infused T cells will provide information about the T-cell mechanisms. This analysis is difficult in inaccessible tissues as opposed to bone marrow or blood cells. It is known that infused T cells migrate throughout the host and reach their target. As a consequence, the effector function of T cells and their distribution to the intended site are crucial. The number of infused cells is only a small fraction of total T cells in the host, in adoptive T-cell therapy. The homing and persistence of infused T cells can be measured by quantitative PCR and/or flow cytometry. Quantitative PCR can be used to obtain information about survival, migration, and homing of infused T cells. Detecting the fate of infused cells by flow cytometry requires reagents that include MHC class I multimers and idiotype-specific antibodies, which can identify CAR-engineered cells. Furthermore, mass cytometry-based platforms and algorithm-driven hierarchical clustering approaches are under development. These techniques will allow information about a vast amount of T-cell markers that will include intracellular proteins, surface markers, RNA species, and phosphoproteins.

T-cell therapy needs to be evaluated on the entire immune response due to its complexity. In a CAR-engineered T-cell immunotherapy trial for treating leukemia, systemic cytokine levels were measured. The results point out that there are strong cytokine-mediated responses that include macrophage-activation syndrome, cytokine-release syndrome, and hemophagocytic lymphohistiocytosis. It was observed that IL-6 was a major cytokine released in response to CAR therapy. Tocilizumab is being used to address this problem.

12.4 Immune Checkpoints

The immune response utilizes several checkpoints or "immunologic brakes" to protect self from the deleterious effects of the over-activation of the immune system against healthy tissue. Cancer cells use these checkpoints to their advantage to escape recognition by the immune system. The monoclonal antibodies directed against two of these checkpoints CTLA-4 and PD-1 (programmed death-1), and their therapeutic use in cancer has already been described. CTLA-4 is abnormally overexpressed on T cells in some forms of cancer that inhibits T-cell response to cancer cells. PD-1 is another immunologic checkpoint that is overexpressed on certain types of cancer cells. It also suppresses the function of T cells that leads to the inability of the immune response to attack the cancer cells. Immunotherapy that will block a checkpoint is expected to result in augmented anti-cancer T-cell response.

Both PD-1 and PD-L1 blockers interfere with the interaction between PD-1 and PD-L1. T and B cells express PD-1 receptors, and their ligand PD-L1 is distributed on a variety of cell types including the cancer cells. Interaction of PDL1 to its receptors inhibits the immune response. Identification of a gene, which is upregulated during T-cell activation and cell death led to the concept that the protein synthesized

by the gene would have clinical value in cancer immunotherapy. It is assumed that the stimulation of the immune response leading to the production of interferon induces PD-L1 expression on cancer cells, which blocks the T-cell response against cancer cells. In this case, induction and inhibition go hand in hand. The rational for this form of immunotherapy is to interfere with the turning off immune checkpoints rather than inducing the immune response. The inhibitors of CTL-4 and PD1/PD-L1 target different points in the immune response. CTLA-4 is a regulator of T-cell activation, whereas PD-1 is a regulator of effector T-cell function. Clinical trials are underway using anti-CTLA-4 and anti-PD-1 to treat prostate cancer, pancreatic cancer, renal cell carcinoma, and non-small cell lung cancer.

Additional coinhibitory molecules on T cells are LAG3 (lymphocyte activation gene 3), BTLA (B and T-lymphocyte attenuator), VISTA (V-domain immunoglobulin suppressor of T-cell activation), and TIM3 (T-cell immunoglobulin and mucin domain-containing protein 3). The development of blocking antibodies to these T-cell co-inhibitors and their potential use for cancer immunotherapy are still in infancy. Using checkpoint inhibitors would be effective against many different types of cancers because they target T-cell-specific molecules and not tumor-specific antigens.

12.5 Combination Therapy

The combination of adoptive cell therapies with checkpoint blockade and/or other therapies is still in infancy. Many combinations are feasible with an expectation of better outcome. For example, the combination of CD40 or 4-1BB, agonistic antibodies, with anti-CTLA-4 or anti-PD-1, checkpoint blockade will enhance the immunopharmacologic effects of adoptively transferred T cells. This will result in the induction of the natural T-cell immune response to the cancer cells. The response of cancer cells to the standard chemotherapeutic agents designed to interfere with the aberrant signaling has a quick onset but a rapid decline, whereas immunotherapies described here have a longer lag phase as well as longer decline. Standard chemotherapeutic drugs that target cancer mutations, such as Bcl-2 inhibitors in lymphoma or Bruton's kinase inhibitors, or BRAF inhibitors in melanoma, in combination with adoptive T-cell therapies, have excellent potential for the cure of various forms of cancer.

12.6 Other Approaches

Development of artificial antigen-presenting cells is an approach for the initiation and sustenance of T-cell response. A T-cell response requires TCR (primary signal) and CD28 receptor (costimulatory/regulatory) signals. Artificial antigen-presenting cells have been developed that lack the ability to deliver the negative co-stimulatory

signal. The monoclonal antibodies directed against human CD3 and CD28 and covalently linked to magnetic beads, as well as artificial antigen-presenting cells, promote proliferation of human naïve and memory helper T cells. In these reproduced T cells, the TCR repertoire is diverse and can be conditioned to release TH1 and TH2 cytokines, dependent on the culture conditions. This artificial antigen-presenting cell does not interact with CTLA-4 and provides a sustained T-cell stimulation. Another approach has been cell-based antigen-presenting cell lines. In this scheme, dendritic cell precursor cell lines are developed that do not express HLA or T costimulatory molecules. However, they secrete cytokines, express adhesion molecules, and possess other features of the dendritic cells. The stimulatory and costimulatory molecules needed for the induction of T cells are added to cell lines by using viral vectors. Additional molecules added include Fc-binding receptors and 4-1BB. These cells are more efficient as opposed to artificial antigen-presenting cells for the survival and proliferation of T cells, antigen-specific T cells, and cytotoxic T cells. Furthermore, they are also capable of inducing helper T cells. The manufacturing protocols for magnetic artificial antigen-presenting cells as well as cell-based antigen-presenting cell lines are similar, beginning with an apheresis product. T cells can also be isolated from peripheral blood lymphocytes, bone marrow, or tumor-infiltrating lymphocytes. The desired subset of cells is cultured in the presence of cell-based artificial antigen-presenting cells expressing the molecules needed for stimulation and costimulation. Culture vessels are used to propagate T cells at high densities, and subsequently the magnetic bead-based antigen-presenting cells are removed. Additional testing is required for the safety of the cells before infusion into the patients.

Additional modification methods include gene transfer in T cells to temporarily or permanently express the therapeutic genes. Other gene therapy-based manipulation of T lymphocytes can administer genes that can enhance many functions of these cells including their survival, proliferation, new antigen recognition, and ability to migrate toward tumor tissue. TCR of known specificity or CAR is needed for redirecting the specificity of the antigen. This requires antigen recognition via antibody-derived complementarity regions and TCR-linked molecule-dependent signaling. Initial immunotherapy trial to treat melanoma with a gene-modified T cells employed retroviral gene transduction in tumor-infiltrating lymphocytes. Redirecting a T cell to an intracellular antigen is best accomplished by transduction of T cells with a specific TCR. Designing a series of TCRs, which are specific for oncogenic driver proteins has an advantage, since most oncogenic proteins are intracellular. This would be a challenge based on the HLA diversity of the patients and the HLA-restricted antigen-specific TCR molecules. The tumor-directed TCRs have low affinity, which explains the difference between the in vitro effects and the poor clinical responses of cancer vaccines, administered alone or in combination with professional antigen-presenting cells.

Several attempts have been made in redirecting polyclonal T cells to an intracellular antigen by using retroviral transduction of native TCRs. This has proved to be very challenging for multiple reasons. The transcription of two different TCRs by T cells will result in four potential TCRs. Multiple approaches have attempted to

resolve this challenge. The clinical trials of native TCR-transduced T cells have shown regression of tumors in some studies, while in others there was on- and off-target toxicity. In some of these studies, TCR specific for MART-1 and MAGE-A3 were used. The toxicity is due to TCR affinity and specificity and not the result of mispairing and poor signaling. Clinical trials are underway utilizing native TCR and engineered TCR-transduced T cells specific for a variety of HLA-restricted antigens to treat leukemias and lymphomas with encouraging results. Other targets being developed include carbohydrate antigen Lewis Y for treating acute myeloid leukemia, multiple myeloma, and myelodysplastic syndromes.

12.7 Additional Comments

To date, the adoptive cell therapy with genetically modified T cells expressing a chimeric antigen receptor (CAR) has failed in patients with solid tumors or low-grade B-cell malignancies that include chronic lymphocytic with bulky lymph node involvement. The success has been limited to B-cell acute lymphoblastic leukemia. Alternative strategies have been applied to boost the antitumor ability of CAR T cells. They include the constitutive expression of CD40 ligand (CD40L, CD154). CD40 is a costimulatory molecule present on antigen-presenting cells, and their activation requires its presence. The antigen-presenting cells are activated when CD154 (CD40L) present on TH cells binds to CD40. T cells when genetically modified to express CD40L exhibit enhanced proliferation of TH1 cells and production of their cytokines. This results in the upregulation of costimulatory molecules (CD80 and CD86), adhesion molecules (CD54, CD58, CD70), HLA class I molecules, HLA-DR molecules, and the Fas-death cell receptor (CD95) on CD40+ tumor cells. As expected, this overexpression of a number of different molecules enhances the immunogenicity of CD40+ tumor cells. Furthermore, these CAR T cells caused production and secretion of IL-12 by monocyte-derived dendritic cells. In studies using animal models, this constitutive expression of CD40L resulted in enhanced antitumor activity in a xenotransplant model of CD19+ systemic lymphoma.

CM-CS1, an autologous CAR T-cell therapy employing NKG2D, has been used. This molecule (NKG2D) is a natural killer cell receptor and is designed to target a number of different cancers due to its wide distribution. This includes both the hematologic cancers and solid tumors. These targets are expressed by many cancers including breast, prostate, and pancreas. Although the current clinical trials being carried out for hematologic cancers include leukemia and myeloma, its broader application would be ideal. The next-generation platform technology is under development that includes combination of CAR T cells with TCR-inhibiting molecules (TIMs). The intention is that TIMs will allow the use of CAR therapy with T cells from healthy volunteers without the risk of graft-versus-host disease (GVHD). The success of TIM/CAR T-cell immunotherapy approach will result in a decrease in the time of treatment and simplify the logistics.

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