Molecular and Integrative Toxicology

George A. Parker *Editor*

Immunopathology in Toxicology and Drug Development

Volume 1, Immunobiology, Investigative Techniques, and Special Studies

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Preface

This book provides a fundamental understanding of immunopathology and immunopathologic processes, with particular attention to nonclinical toxicology studies. Chapters provide an overview of general immunobiology, cells of the immune system, signaling and effector molecules, and immunopathology assays. A companion volume*, Immunopathology in Toxicology and Drug Development: Volume 2, Organ Systems*, offers summaries of organ-specific immunobiology and immunopathology as well as common responses to xenobiotics.

These informative and strategic books were created in response to the large segment of drug development that focuses on chronic diseases, many of which involve alterations to the immune system. Therapies that target these diseases commonly involve some form of immunomodulation. As a result, the two volumes of *Immunopathology in Toxicology and Drug Development* are critical texts for individuals involved in diverse aspects of drug development. Readers will acquire a thorough understanding of immunopathology for detection and accurate interpretation of pathologic effects of xenobiotics on the immune system.

Durham, NC — George A. Parker

Contents

About the Editor

George A. Parker, a leading expert in the field of toxicologic pathology, is currently a Senior Scientific Director, Global Pathology, at Charles River Laboratories. Dr. Parker is a graduate of Auburn University College of Veterinary Medicine and completed a residency program in veterinary pathology at the Armed Forces Institute of Pathology, as well as a Ph.D. program in molecular and cellular immunology at the University of Medicine and Dentistry of New Jersey. He is a board-certified pathologist and toxicologist as well as a fellow of the distinguished International Academy of Toxicologic Pathology. He has more than 35 years of experience in the field of toxicologic pathology, with an emphasis on the nonclinical safety assessment of drugs, agricultural chemicals, and industrial chemicals. Dr. Parker's areas of specific expertise include immunopathology, toxicologic pathology of juvenile animals, and radiation pathology.

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Chapter 1
Basic Immunobiology

George A. Parker and Tracey L. Papenfuss

Abstract Familiarity with basic immunobiology has long been necessary for the interpretation of xenobiotic-related changes in non-clinical toxicology studies. However, that requirement has become even more critical in the current era of biopharmaceutical development, wherein the candidate drugs may cause immunomodulation as a desired pharmacological endpoint. In these latter studies the toxicologic pathologist and toxicologist must distinguish between direct pharmacological effects, excessive pharmacological effects, off-target effects, or effects that are secondary to the intended pharmacological effects. In many studies the pathology observations are complicated by stress influences on the test animals, and in juvenile toxicology studies there is the additional variable of postnatal organ development. The aim of this chapter is to provide toxicologists and toxicologic pathologists with a brief overview of the salient immunologic concepts, structures, cells and physiologic processes. Current review-type references are provided for further insight into the various components of this immense topic.

Keywords Immunobiology • B cells • T cells • Macrophages • Neutrophils • NK cells • Eosinophils • Basophils • Memory cells • Effector cells • Signaling molecules

1.1 Introduction

Experience suggests immune system perturbations occur in association with a large percentage of xenobiotic-induced pathologic processes. These may take the form of inflammatory reactions generated by the innate immune system in response to xenobiotic-associated tissue injury, direct modulation of the adaptive immune system by xenobiotics, or secondary modulation of the adaptive immune system due to

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systemic stress that results from various forms of xenobiotic-associated injury. Immune system injury may increase the propensity to develop neoplasia and, conversely, appropriate stimulation of the immune system may have the desirable pharmacological end-point of retarding the development and growth of neoplasms. Some neoplasms have developed mechanisms for combating the immunologic mechanisms that hinder their growth, and blocking those tumor-associated compensatory mechanisms by pharmacologic intervention could have a beneficial effect for the host. The broad-based involvement of the immune system in non-clinical toxicology studies requires familiarity with immunology and immunopathology as these potentially complex studies are designed and interpreted.

1.2 General Purpose, Function and Characteristics of the Immune System

Defense of the primary organism, whether it be human, rat, fish or worm, is a major concern of all life forms. If one accepts the endosymbiont hypothesis, which postulates that mammalian mitochondria are derived from bacteria that invaded our ancient, single-celled ancestor, then it appears this need for self-defense has been with us for some time. The innate immune system is the original protection against invasion. The adaptive immune system evolved largely to resolve failures in the innate immune system, which resulted in penetration of external agents into the confines of the primary organism. The early stages of immunology investigation were focused on the adaptive ('lymphocyte/macrophage') system but, more recently the biomedical community has come to recognize the importance of the innate immune system. Rather than consisting of nonspecific inflammation and simple physical and physiological barriers to infection, the innate immune system is now known to involve receptors, signaling pathways and effector molecules that equal the complexity of the adaptive immune system. The innate and adaptive immune systems effectively cooperate to provide protective responses against pathogenic insult and maintain tissue homeostasis.

In this chapter the components of the immune system, immune responses in health and disease, approaches to evaluating immune responses and implications of immune alterations relevant to drug development will be evaluated.

1.2.1 Phylogeny of the Immune System

Defense against invading pathogens, which is a necessary component of any organism's biology, involves a system of organs, cells and physiological processes that serve to protect the host from disease, maintain homeostasis and ensure survival.

Immune responses vary in complexity, but it is generally considered that the more complex the host organism, the more complex that organism's immune responses will be.

Organisms have developed a complex system to protect themselves from pathogens. While we are most familiar with the immune system of mammals, rudimentary immune responses exist even in unicellular prokaryotes (Buchmann 2014; Vinkler and Albercht 2011). Specifically, prokaryotes have clustered regularly interspaced palindromic repeats (CRISPRs) which can degrade invading foreign proteins and, along with metazoans, possess receptors on their cells which can differentiate self from non-self. As one moves up the scale in evolutionary complexity, organisms have an increasingly complex armamentarium of immune responses. Invertebrates such as sponges, molluscs, crustaceans, insects, echinoderms, etc. have phagocytic cells that have functions similar to the macrophages of higher vertebrates. These invertebrate cells possess pattern-recognition receptors (PRRs) that recognize the conserved molecular structures of 'pathogen-associated molecular patterns; PAMPs' expressed on or by various infectious agents (e.g., viruses, bacteria, fungi, protozoans and helminths). The evolutionarily conserved PRRs such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are expressed on macrophage-like phagocytes in sea sponges and several cell types (e.g., macrophages, dendritic cells, neutrophils, eosinophils and basophils) of higher vertebrates (Buchmann 2014). Such cells comprise a portion of the non-specific innate immune response, and the TLRs and NLRs on these cells play an important role in protecting an organism from pathogens and helping maintain homeostasis by promoting a downstream reaction that can directly attack and kill the organism or, in higher vertebrates, interact with a more sophisticated and specific adaptive immune response.

A higher degree of sophistication in immune responses is seen in more complex organisms, such as cartilaginous and bony fishes, amphibians, reptiles, birds and mammals. These organisms developed an increased ability to differentiate self from non-self by development of major histocompatibility complexes and have developed adaptive immune responses (e.g., T and B lymphocytes) to assist innate immunity and provide more robust immune responses (Hirano et al. 2011). Lymphocyte-based adaptive immunity is present in urochordates (tunicates) but it is only with the appearance of jawless vertebrates that lymphocytes bearing some complexity of diversified adaptive immunity is seen (Hirano et al. 2011). It is with jawed vertebrates that many aspects of the sophisticated components comprising the adaptive immune response (e.g., MHC, T cell receptors, recombinase-activating genes; RAG1/RAG2) are seen, although much recent research has shed light on the evolutionary development of adaptive immunity in jawless vertebrates as well (Hirano et al. 2011). These additional components of immune responses allow specificity of responses to pathogens, immunological memory and enhanced secondary responses that can more effectively combat infectious challenge. Cells of both the innate and adaptive immune response utilize a wide array of effector cells, receptors, signaling and effector molecules (e.g., cytokines, chemokines and other soluble mediators) to produce a highly effective immune response, the details of which will be introduced in this chapter.

1.2.2 Overview of Organs and Tissues of the Immune System

Numerous immune cells circulate throughout the body and populate both immune and non-immune organs. Organs that classically have a predominant immune function are categorized into primary, secondary and tertiary lymphoid organs, and it is at these sites that many immune interactions are developed. Initial engagement with pathogens often occurs at the site of contact with the environment, which is most commonly the skin or mucosal surfaces, but essentially all organs throughout the body participate in immune responses in some capacity.

In addition to immune functioning in the primary, secondary, or tertiary lymphoid structures, there is a broad category of immunological functions that take place in organs which are not traditionally considered to be part of the immune system. The latter category could include virtually all organs and tissues of the mammalian body, as most organs and tissues have some role in resistance to pathogens or other forms of damage. The present discussion will be limited to the more salient examples of immune system function in non-immune organs. In-depth presentations of the structure and function of immune system organs are included in the respective chapters for those organs. Details of embryogenesis/organogenesis and senescence are presented in Chaps. 4 and 5, respectively. The aim of this summary is to provide an overview that links similar functions in the various immune system organs.

1.2.3 Primary Immune System Organs

Primary immune system organs are those organs which develop fully in response to genetically determined directives, with no requirement for environmental influence. The bone marrow and thymus are considered to be the primary immune system organs in mammals, with bursa of Fabricius being the bone marrow equivalent in avian species. The liver serves as the primary hematopoietic organ in fetal mammals and serves as a primary immune system organ, but that role is transferred to the bone marrow commencing near the time of birth. Large, prenatally developed Peyer's patches in sheep and other ruminant animals meet some of the requirements for classification as primary immune system organs, but in this discussion the Peyer's patches of all species will be considered as secondary lymphoid organs.

The bilobed thymus forms from the branchial arches early in fetal development, and the organ comes to rest in the anterior mediastinal space by the time of birth. The organ has a highly cellular cortex that is the site of proliferation and immunological selection of T lymphocytes derived from naïve T cell precursors which are received from the liver (prenatally) or bone marrow (postnatally). The less cellular thymic medulla is involved in maturation and release of T lymphocytes. The thymus is one of the more dynamic and sensitive organs in the mammalian body, exhibiting a continuous massive level of cortical lymphopoiesis. Due to positive and negative selective processes, fewer than 5% of the thymocytes that are generated in the thymic cortex eventually exit the thymus to function as circulating T cells (Fig. 1.1).

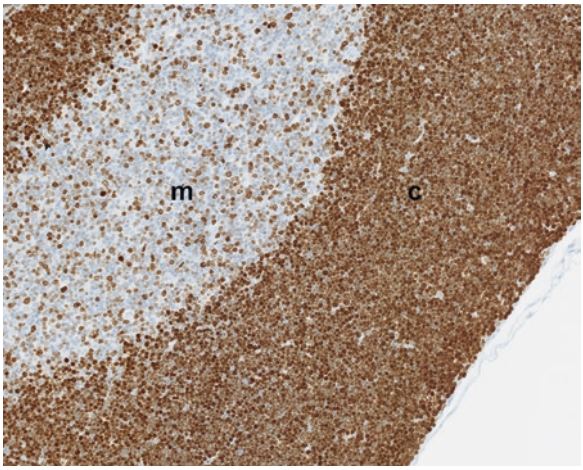

Fig. 1.1 An immunohistochemical stain for proliferating cells performed on the thymus of a young rat reveals a massive number of positively stained (*brown*) cells in the cortex (c), with fewer positively stained cells in the medulla (m). This image demonstrates the relative rate of cellular proliferation in the thymic cortex versus the medulla. Immunohistochemical stain for Ki67 proliferation marker, with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain. 15× objective magnification

The non-surviving cells die of apoptosis and the resultant cellular debris is quickly removed by tingible-body macrophages. The thymus is exquisitely sensitive to xenobiotics that affect rapidly dividing cell populations, and developing thymocytes at the double-positive (DP) stage are equally sensitive to stress-related or experimentally administered glucocorticoids (Fig. 1.2). Histologic changes in the thymus are very common manifestations of immunological perturbations in non-clinical toxicology studies.

1.2.4 Secondary Immune System Organs

Secondary immune system organs have a genetically determined anatomical location and structural basis, but development of the histomorphologically 'normal' organs is dependent to some degree on immunological responses to environmental influences. The major secondary lymphoid organs that are routinely sampled in non-clinical toxicology studies are the spleen, lymph nodes and Peyer's patches of the small intestine. Other secondary organs include various organ-associated presentations of mucosa-associated lymphoid tissue (MALT) as well as the various types of gastrointestinal immunological structures such as the diffuse lymphoid cell population of the intestinal mucosa, cryptopatches, solitary lymphoid follicles and lymphocyte-filled villi of the small intestine, and lymphoepithelial complexes of the large intestine. As presented above, some forms of MALT in individual species have

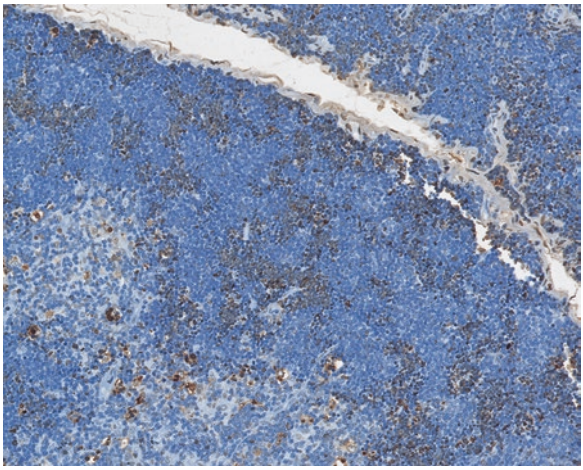

Fig. 1.2 The TUNEL (Terminal deoxynucleotidyl transferase dUDP Nick End Label) staining on a rat thymus following experimental administration of a corticosteroid reveals areas of brown-stained apoptotic cells with intervening areas of unstained, non-apoptotic cells. This image demonstrates the regional congregations of thymocytes that are at similar stages of development. The apoptotic thymocytes in this image are most likely at the double-positive (DP) stage, during which the cells are exquisitely sensitive to corticosteroids. The non-stained cells represent thymocytes which are at a corticosteroid-resistance stage of development. Note the apoptotic and non-apoptotic cells exist in clusters, as opposed to being randomly intermixed. This is a visual manifestation of the shared state of development of physically contiguous clusters of thymocytes, which resulted from proliferation of individual naïve thymocytes after they entered the thymus. TUNEL staining with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain. 20× objective magnification

features of primary lymphoid tissue. Some forms of MALT, e.g., bronchus-associated lymphoid tissue (BALT) of mice, are expressed only in response to inflammation, thus could be considered tertiary rather than secondary lymphoid tissue. In these situations an intermediate term such as 'inducible BALT' (iBALT) may be used for MALT structures that are formed only in response to stimuli.

1.2.5 Tertiary Lymphoid Tissue

Tertiary lymphoid tissue, or ectopic lymphoid-like structures (ELS), consists of lymphoid cell accumulations found at sites of inflammation in non-lymphoid organs or tissues. Ectopic lymphoid tissue may exhibit definitive features of lymphoid tissue organization such as follicle and germinal center formation (Fig. 1.3). As described in Volume 1, Chap. 4, the development of secondary immune system organs is dependent on signaling by a number of pro-inflammatory cytokines that influence lymphoid tissue initiator cells (LTi) of liver or bone marrow origin and lymphoid tissue

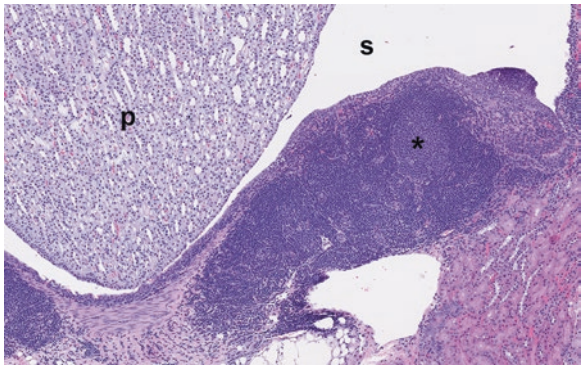

Fig. 1.3 Kidney of a non-dosed mouse with chronic inflammation and densely cellular tertiary lymphoid tissue in the renal pelvis. Note the prominent germinal center (*) in the tertiary lymphoid tissue. *p* = renal papilla, *s* = renal sinus. H&E stain, 10× objective magnification

organizer (LTo) cells that originate from, or have a shared origin with, pre-adipocytes. All these necessary factors are present in areas of chronic inflammation, and result in the formation of tertiary lymphoid tissue In the animal species that are commonly used in non-clinical toxicology studies, tertiary lymphoid tissue is commonly seen in association with nonspecific or immune-mediated chronic arthritis, chronic cystitis such as that associated with uroliths, immune-mediated thyroiditis (particularly in beagle dogs), and nonspecific chronic inflammation in the subcutis or abdominal cavity.

Presence of tertiary lymphoid tissue has long been recognized, but has been largely considered as incidental to the inflammatory process. Emerging information suggests the development and function of tertiary lymphoid tissue may be of major importance in local resistance to infection and tumors, but may also have a significant deleterious effect when part of an autoimmune disease process. Tertiary lymphoid tissue is thought to coordinate endogenous antitumor immune responses (Coppola et al. 2011; Gu-Trantien et al. 2013; Di Caro and Marchesi 2014), and its presence within tumors has been shown to be a positive prognostic feature of a variety of human tumors (Di Caro et al. 2014; Dieu-Nosjean et al. 2008; Gu-Trantien et al. 2013; Messina et al. 2012). However, presence of tertiary lymphoid tissue within tumors is not universally positive with regard to disease progression. In a mouse hepatocellular carcinoma model that displayed abundant tertiary lymphoid structures, the lymphoid structures were found to constitute a protective niche for malignant hepatocyte progenitor cells (Finkin et al. 2015).

Development of tertiary lymphoid tissue may be pharmacologically influenced to reduce autoimmune diseases or promote anti-tumor immunity. A protein known as LIGHT stimulates the lymphotoxin receptor that is primarily involved in formation of tertiary lymphoid tissue. Linking LIGHT with an antibody to certain tumor cells promoted the formation of tertiary lymphoid tissue within tumor masses, which potentiated the activity of a checkpoint inhibitor-type chemotherapeutic agent and essentially eliminated the neoplasms (Tang et al. 2016).

See (Buckley et al. 2015) for a more extensive dissertation on tertiary lymphoid tissue.

1.2.6 Non-immune Organs with Non-specific and Specific Immunologic Functions

Defense against invading pathogens or response to injury is critical to survival of the organism, therefore it is not surprising that many, perhaps most, cells and organs have some immunologic function in the broad sense. A combination of physical and chemical barriers as well as an array of innate immune cells are commonly located at the interface between host and environment. Epithelial surfaces are the primary physical barrier that can prevent pathogenic organisms from entering the body. The skin provides a protective barrier and is comprised of a combination of cells (keratinocytes and immune cells), lipids, antimicrobial compounds and a local microbial environment (i.e., microbiota). The epithelial linings of the respiratory, gastrointestinal, urogenital, ocular and mammary organ systems serve a similar barrier function. In addition to posing a simple physical barrier, many of the epithelia contain cells and produce molecules that afford additional protection to the host organism.

In addition to immune cells strategically located throughout mucosal surfaces, the epithelial sites provide protection through a combination of secretions (e.g., mucus or tears), clearance mechanisms (e.g., peristalsis in the gastrointestinal tract, mucociliary clearance in the respiratory tract, etc.), protective local microbial flora (microbiome), and soluble factors including antibodies, interferons, complement and anti-microbial compounds (e.g., defensins, canthelicidins, etc.). The acidic pH of the stomach helps to sterilize ingested material. The mucociliary blanket of the respiratory tract serves to capture potentially injurious particles or organisms, and transports them up the trachea for subsequent expulsion. Intestinal peristalsis serves to propel potentially harmful organisms and materials through the gastrointestinal tract for eventual elimination. Intestinal peristalsis acts in concert with the abundance of divalent IgA that is released into the intestinal lumen, which binds microbes into clusters that are less able to invade tissues of the intestinal wall and are more amenable to elimination via intestinal peristalsis. Ureteral peristalsis serves to 'pump' urine from the renal pelvis into the ureter. Presence and movement of the fluids and lipids that constitute tears serve to protect the eye from some forms of injury and conduct potentially injurious particles or agents away from the exquisitely sensitive globe. Interruption of any of these physiological protective mechanisms can have a disastrous effect on the organ or the organism as a whole. Most of the processes that involve physical movements are dependent on intact neurological function, thus neuroactive xenobiotics may have a serious detrimental effect on overall immune system function.

In addition to the skin and mucosal epithelial sites, the liver has a number of important functions in innate and adaptive immunity, and perturbations in hepatic structure or function can result in significant ramifications in both the innate and adaptive immune systems. Contributions to the innate (nonspecific) immune system include production of acute phase proteins, nonspecific phagocytosis of particles, nonspecific pinocytosis of molecules, and nonspecific cell killing. Hepatic involvement in innate immunity contributes to the systemic response to local inflammation, clearance of particles and soluble molecules from the circulation, and killing of invading cells such as neoplastic cells. The liver is the most common site of

xenobiotic-associated tissue injury (Horner et al. 2013), therefore the possibility of liver-related alterations in immunological function is of particular concern in non-clinical toxicology studies. A more extensive presentation of liver involvement in immune system functions is presented in Volume 1, Chap. 2.

Downstream effects of liver involvement may impact on a number of parameters that are typically analyzed in toxicology studies. For example, fibrinogen levels may be increased because fibrinogen is an acute phase reactant that is produced as a generalized response to inflammation. Albumin is a negative acute phase reactant, which means its production is reduced in response to inflammation. Albumin serves as a calcium-binding protein in rats, therefore, lower circulating albumin levels may result in lower serum calcium levels. Albumin levels are determined by direct analysis in typical clinical chemistry screens, as is total serum protein level. Serum globulin level is a calculated value (total protein minus albumin = globulin), as is the albumin/globulin ratio. In summary, the liver's contribution to the acute phase reaction, which is particularly common in infusion studies, can result in increased fibrinogen level, decreased albumin level, lower serum calcium value, and altered calculated values for globulin and albumin/globulin ratio. These alterations which are associated with the experimental manipulation must be differentiated from direct effects of xenobiotic administration.

The liver is the primary site of hematopoiesis in the fetus, thus has a major role in the formation of cells of the adaptive immune system. Liver involvement in the adaptive (specific) immune system of adults includes deletion of activated T cells, induction of tolerance to ingested and self-antigens, extra-thymic proliferation of T cells, and deletion of many of the signaling and effector molecules associated with immunity and inflammation. Hepatic involvement in adaptive immunity allows clearance of activated T cells and signaling molecules following inflammatory reactions, and promotes immunologic tolerance toward potentially antigenic proteins that are absorbed from the intestinal tract. Extra-thymic T cell development in the liver assumes increasing significance in aged mammals following thymic involution**.**

Whether responses occur in non-immune or classical immune organs, various components of the immune system work together to develop an immune response. The following sections will highlight some of the important aspects of the coordination between the innate and adaptive immune responses and the resultant outcome of those interactions.

1.3 Innate Immune System Overview

The innate immune response is an early line of defense and rapid response to insult or intrusion by infectious agents. It is comprised of a combination of physical and chemical barriers and a wide array of immune cells. Though innate immune response occurs rapidly, it is non-specific, limited in diversity, and has no ability to generate memory responses. The primary components of the innate immune response are (1) physical and chemical barriers (e.g., skin, epithelium, antimicrobial peptides), (2) immune cells with phagocytic or non-specific cytotoxic capabilities (e.g., neutrophils, macrophages, dendritic cells, and natural killer cells), and (3) the complement

system and other blood-borne mediators of inflammation. There are specific cells within the innate arm of the immune response that bridge innate and adaptive immune responses. Professional antigen-presenting cells such as dendritic cells and macrophages are able to rapidly respond to invading organisms and acquire specific antigenic components (e.g., peptides) which they present to cells of the adaptive immune to generate a more specific immune response that includes immunological memory, thus creating the potential for a hastened and enhanced amnestic response upon second exposure to the pathogen. There are additional non-specific components including secreted compounds and cellular interactions that further allow for integration across the innate and adaptive immune responses.

The adaptive immune response serves as a refinement of the immune response that exhibits immunologic specificity and can contribute to the generation of an amnestic (memory) response. Macrophages and DCs, which are typically categorized as members of the innate immune system, play an important role in coordinating responses between innate and adaptive immune responses. These APCs express MHC class II molecules to modulate $CD4^+$ T cell responses which subsequently influence multiple arms of adaptive immunity (i.e., both cell-mediated and humoral immunity). Refinement of various signals (e.g., TLRs, NLRs or other environmental factors including hormones and cytokines) results in the expression of specific co-stimulatory molecules and cytokine profiles which enhance the differentiation of helper T cell subsets (Cools et al. 2007). APCs also directly interact with lymphocytes, the effector cell populations of the adaptive immune response.

1.3.1 Signaling Pathways of the Innate Immune System

Initiation of the innate immune response involves activation of evolutionarily conserved pattern recognition receptors (PRRs) on mammalian phagocytes (Philpott and Girardin 2004). Inflammation-producing agents may be foreign molecules such as pathogens, which have a pathogen-associated molecular pattern (PAMP), or may be endogenous molecules that are inappropriately expressed or mislocated (damage-associated molecular pattern, or DAMP). Initial receptor recognition of the molecular patterns of organisms is highly context-sensitive, with similar molecular patterns being treated as 'normal' or pathologic depending on location of the signaling event and existence of additional signals of 'danger'. The PAMPs and DAMPs are recognized by innate immune receptors that either induce or inhibit an immune response (Medzhitov and Janeway 2002). Whether an inflammatory response is subsequently generated in response to the signals is also dependent on the context of the initial signaling event (Silke et al. 2015).

There are four main families of PRRs: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-1-like receptors (RLRs), and NOD-like receptors or nucleotide oligomerization domain/leucine-rich repeat-containing receptors (NLRs). Extracellular pathogens are detected primarily by members of the TLR family, while the remaining families of PRRs are focused primarily on intracellular pathogens and cellular constituents. Regardless of whether the signal is intra- or

extracellular, the receptors use similar intracellular signaling pathways such as NF-κB and Fos–Jun to initiate the transcription of inflammatory cytokines and chemokines.

The Toll receptor of *Drosophila* fruit flies is a member of the homeobox group of genes that control major organizational structures. Early investigations revealed that some mutations in Toll receptors resulted in increased susceptibility of the fruit flies to infectious organisms such as *Aspergillus fumigatus*. The cytoplasmic signaling domain of the Toll receptor was found to be homologous to the mammalian IL-1 receptor. Additional studies revealed the existence of mammalian proteins that were involved in activation of innate immunity in mammals, thus were named 'Toll-like receptors' (TLRs).

The TLRs are membrane-spanning proteins that have a shared feature of leucine-rich repeats (LRRs) in the extracellular domain and an internal signaling domain that resembles IL-1 receptor (Takeda and Akira 2004). Of the 13 families of TLRs identified to date, families 1–10 are conserved in both mice and humans and families 11–13 are expressed only in mice. TLRs are located on cell membranes, which may be internalized into phagosomes, and on the membranes of endosomes and lysosomes. Each TLR family has a repertoire of specificities for certain pathogen-associated molecular patterns (PAMPs) associated with microbial pathogens and damage-associated molecular patterns (DAMPs) associated with damaged tissues. TLRs that recognize extracellular pathogens are concentrated on the plasma membrane, while those recognizing intracellular constituents are concentrated on the membranes of endosomes/lysosomes. TLR4, which has binding specificity for the lipopolysaccharide (LPS) of gram-negative bacteria, moves from plasma membrane to endosomal/lysosomal membranes upon binding of lipopolysaccharide (LPS).

TLRs typically dimerize upon binding of a specific PAMP or DAMP, and this binding initiates intracellular signaling pathways that activate numerous immunological processes. The downstream signaling initiated by TLR dimerization is mediated by adaptor proteins that recognize the TIR (Toll/IL-1 receptor) cytoplasmic domain of the TLR. Two key adaptor proteins are MyD88 (myeloid differentiation factor 88) and TRIF (TIR-domain-containing-adaptor-inducing IFN-B factor). One major downstream signaling pathway that is shared between TLRs is NFκB, which is a major immunity-related pathway that induces numerous genes for many immunologically important molecules, including defensins, iNOS, chemokines, and pro-inflammatory cytokines. Other TLRs activate downstream signaling pathways for gene products which are specific for resistance to a particular category of PAMP/DAMP. The intracellular TLRs that recognize viral components induce the synthesis and secretion of type I interferons, which serve to inhibit viral replication.

The C-type lectin receptors (CLRs) are a second group of pathogen recognition receptors (PRRs) that are expressed on cells of the innate and adaptive immune systems. CLRs recognize the carbohydrate components of fungi, mycobacteria, viruses, parasites and some allergens. Some CLRs function as phagocytic receptors, and all trigger signaling pathways that activate specific genes. CLR-related signaling pathways may have pro-inflammatory or anti-inflammatory end-products, and may collaborate with or oppose the end-products of signaling by other PRRs such as TLRs.

The RIG-like receptors (RLRs) are soluble cytosolic proteins that are critical sensors of viral infection. RLRs such as RIG-1, MDA5 and LGP2 are able to distin-

guish between viral RNA and cellular mRNA (Abbas and Lichtman 2015). Upon activation, RLRs undergo a conformation change that leads to downstream signaling and production of interferons α and β as well as antimicrobials, chemokines and pro-inflammatory cytokines.

The NLRs are a large family of cytosolic proteins that are activated by PAMPs and DAMPs. All have an LRR domain and a nuclear-binding domain. The NLR proteins are divided into three groups based on their domain structure: NLRC with caspase recruitment domain (CARD), NLRB with baculovirus inhibitory repeat domains, and NLRP with pyrin domains. NOD1 and NOD2, well-known examples of the NLRC family, recognize breakdown products resulting from the synthesis or degradation of cell walls of intracellular bacteria, or extracellular bacteria that penetrate into cells. NOD1 and NOD2 are present in the cytoplasm of epithelial cells and many cells of the immune system (Inohara and Nunez 2003). Certain NLRs complexed with proteins such as proteases are termed 'inflammasomes', which have a central role in the induction of IL-1.

Tumor necrosis factor (TNF) and the IL-1 family of cytokines are potent modulators of inflammation that are produced in very small quantities except in response to DAMPs and PAMPs. IL-1 exists in the cell as an inactive pro-IL-1 precursor protein that is acted upon by caspase-1 (formerly known as IL-1 Converting Enzyme, or ICE) to form the active IL-1 cytokine. In order to achieve activation of pro-IL-1, pro-caspase-1 must be activated and incorporated into a multiprotein aggregate that is known as the inflammasome. Three NLRs (NLRP1, NLRP3, and NLRC4) are known to form inflammasomes that activate pro-caspase-1, which in turn acts on pro-IL-1 to form active IL-1. NLRP3, probably the best-characterized of the inflammasomes, is expressed by macrophages, monocytes, neutrophils, dendritic cells, lymphocytes, and some epithelial cells. It is activated by a broad spectrum of pathogens such as bacteria, fungi, and some viruses, and by components of damaged tissue such as β amyloid (e.g., in the plaques of Alzheimer's disease), components of extracellular matrix such as hyaluronic acid, crystals such as urate crystals in gout patients, inhaled silica or asbestos, and metabolic molecules such as ATP and glucose.

1.3.2 Natural Antibodies

Antibodies are traditionally considered to be a refined and specific response generated during an adaptive immune response. However, even in germ-free environments with no possible pathogen exposure, vertebrate animals produce 'natural antibodies' which are germline-coded antibodies produced without previous specific antigen exposure. These natural antibodies, along with the maternal immunoglobulin that is transported from the dam across the placenta into the fetus late in gestation, provide immediate protection of the newborn that lacks immunological resources and has not had sufficient exposure time to mount a traditional adaptive immune response. In some ways natural antibodies constitute a link between the

innate and adaptive immune systems (Carsetti et al. 2004), in that their protection relies on the immunoglobulin molecules similar to those employed by the adaptive immune system, yet the generation of the immunoglobulin molecules is determined by evolutionary genetics rather than previous exposure of the individual to specific antigens.

Natural antibodies are primarily of the immunoglobulin M (IgM) isotype, with a smaller proportion being IgG or IgA isotype. They are produced by a distinct subset of B cells known as B-1 cells, which produce antibody without any requirement of prior antigenic stimulation. B-1 cells are the predominant B cell type in the fetus and neonate, but after birth the B-1 cell population declines and is replaced by the traditionally recognized B cell population of adults. Nonetheless, a small population of B-1 cells remains in the spleen and wall of the intestine, and can be recovered by peritoneal washes performed on adult mice. The B-1 cells that produce natural antibodies are found in the marginal zones of the spleen rather than lymphoid follicles, therefore natural antibodies do not undergo the affinity maturation process that occurs in germinal centers.

Natural antibodies are encoded by germline variable region (V_H and V_L) genes that have stable, restricted repertoire and reactivity. Natural antibodies are often polyspecific, thus react with structurally dissimilar endogenous and/or exogenous antigens (e.g., histones, polynucleotides, polysaccharides, or immunoglobulins). Natural antibodies are typically produced in response to self- and non-self antigens composed of phospholipids, carbohydrates, single-stranded DNA, peptides (e.g., amyloid beta peptide) or surface glycoproteins (e.g., CD90). Some natural antibodies recognize "oxidation-specific" epitopes generated during oxidative processes in metabolism, aging, and inflammation, including atherosclerosis, and act as PRRs for these molecular structures (Schwartz-Albiez et al. 2009). The oxidation-specific epitopes are highly conserved, occurring on microbes as well as aging and apoptotic host cells, thus represent a class of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) that are recognized by receptors of the innate immune system. Natural antibodies can recognize some of the polysaccharide molecules expressed on the surface of tumor cells, thus can function in tumor immunity (Schwartz-Albiez et al. 2009).

Natural antibodies play an important role as a first line of defense against bacterial pathogens, but also function in the removal of effete intracellular molecules or cell surface structures of dying cells. In this latter function natural antibodies are important in preventing autoimmunity and inflammation that could result from reactions to cellular components.

1.3.3 Complement

Complement is one of the oldest known components of immunity, both phylogenetically (Nonaka and Yoshizaki 2004) and medically, and has a central role in the ability of the innate immune system to detect and destroy pathogens (Walport

2001a, b; Morgan 2000; Ricklin et al. 2010; Sarma and Ward 2011). The complement system consists of circulating pro-enzyme proteins that eventually constitute the effector molecules, and cell membrane-bound proteins that constitute the activator elements. The circulating proteins normally exist in an inactive status, which can be activated by the classical, alternative and lectin pathways (Walport 2001a, b; Morgan 2000; Ricklin et al. 2010; Sarma and Ward 2011). Complement activation by any pathway results in the formation of convertase enzyme complexes that cleave circulating C3 into C3a (anaphylatoxin) and C3b (opsonin) and C5 into C5a (anaphylatoxin) and C5b. Deposition of C5b on a target cell membrane initiates the membrane attack complex (MAC), which punches a hole in the target cell membrane and causes lysis of the target cell. Opsonins coat pathogens and facilitate their uptake by phagocytic cells. Anaphylatoxins activate neutrophils, monocytes and mast cells, all of which contribute to the innate immune response (Walport 2001a, b; Morgan 2000; Ricklin et al. 2010; Sarma and Ward 2011).

Experimental evidence indicates that complement has functions outside of the innate immune system. The complement receptors that are present on virtually all cells, including cells of the adaptive immune system, have myriad functions that include sequestration of antigenic proteins into the iccosomes of follicular dendritic cells, activation of $CD4^+$ and $CD8^+$ T_H cells, reducing the threshold for B cell activation, and maintenance of B cell tolerance and memory (Carroll 2004a, b; Carroll and Isenman 2012; Strainic et al. 2008; Kemper and Kohl 2013; Clarke and Tenner 2014). In addition to detection and destruction of pathogenic organisms, complement also aids in the destruction of effete or defective self-molecules, which may explain the elevated incidence of autoimmune disease in association with complement deficiencies (Mayilyan 2012; Lewis and Botto 2006; Peerschke et al. 2004; Chen et al. 2010).

While complement has historically been considered as a serum protein of liver origin, it is now clear that bone marrow is an additional major source of serum complement (Morgan and Gasque 1997). Virtually all cells can produce complement (Kolev et al. 2014), and in sites such as the brain and eye, local production is the main source of complement (Barnum 1995; Naughton et al. 1996). Complement activation has historically been considered an extracellular event, but it is now known that intracellular complement activation also occurs. Various cell types have intracellular accumulations of C3 and activating, cell type-specific proteases stored within endosomal and lysosomal compartments (Liszewski et al. 2013).

The location of complement activation has a major influence on the subsequent downstream events. Activation of serum complement results in complement fragments that directly lyse pathogens, opsonize pathogens to facilitate phagocytosis, and recruit/activate cells of the innate and adaptive immune systems (Kolev et al. 2014). These actions of circulating complement represent an ‘endocrine-type’ effect, wherein the biological action takes place some distance from the site of origin of the effector molecules. By contrast, intracellular complement activation is an ‘autocrine-type’ effect, wherein the biological action takes place within the site/cell of origin of the effector molecules. Intracellular complement activation may explain the rapid (within minutes) appearance of C3 fragments on the surface of activated T cells (Liszewski et al. 2013; Cardone et al. 2010).

1.3.4 Cells of the Innate Immune System

Immune cells of the innate immune response include natural killer cells, mast cells, phagocytic cells (including dendritic cells and macrophages) and granulocytes (including eosinophils, basophils and neutrophils). These cells help eliminate pathogens and are non-specific in their actions. Dendritic cells and macrophages, along with B-cells, serve as antigen-presenting cells (APCs) and serve as an important bridge between innate and adaptive immune responses.

Granulocytes: Granulocytes are immune cells which can be characterized based on specific functions and identified based on the staining characteristics of intracellular granules with specific stains. As part of the innate immune system, granulocytes have a rapid and non-specific response to pathogens and other stimuli. Neutrophils, eosinophils, basophils and mast cells all contain cytoplasmic granules, but the term 'granulocyte' is typically restricted to neutrophils, eosinophils and basophils. Granulocytes mediate much of their action through the production and release (degranulation) of toxic materials that are stored within their granules. Depending on the cell population, these granules can contain various antimicrobial agents (defensins & eosinophil cationic protein), enzymes (acid hydrolases, lysozyme), NADP oxidase, vesicles with low pH, nitric oxide, superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, and hypohalite.

Neutrophils, the most abundant immune cell population in the blood, have the role of patrolling the body. As their name implies, neutrophils have granules with neutral-staining characteristics. The IL-23/IL-17/G-CSF feedback loop is a primary mechanism controlling neutrophil production and is a link between adaptive T_H17 responses and the innate immune system (reviewed in (Mayadas et al. 2014). Following production in the bone marrow, neutrophils circulate and, with appropriate chemokine signals, traffic to sites where they can adhere to endothelial cells (i.e., marginate) and extravasate into the tissue (Kruger et al. 2015). This well-defined classic multistep adhesion cascade involves attachment (capture), rolling along the endothelium, first arrest with cell spreading, crawling along the endothelium and transmigration into the tissue either by a paracellular or transcellular route (Mayadas et al. 2014). Neutrophils are a first line of defense to bacteria and directly attack these microorganisms by phagocytosis (ingestion followed by of granule proteins which contain anti-microbial and bactericidal compounds), generation of reactive oxygen species, release of various granule components (i.e., degranulation) and the formation of neutrophil extracellular traps; NETs (Mayadas et al. 2014; Kruger et al. 2015; Brinkmann et al. 2004). Neutrophils can be activated by numerous mechanisms including pattern-recognition receptors (e.g., pathogen-associated molecular patterns; PAMPs, damage-associated molecular patterns; DAMPs), opsonic receptors (e.g., IgG and C3b, pentraxins, FcyRIIB) and various G protein-coupled receptors (e.g., IL-8, C5a and adenosine) (Mayadas et al. 2014). Neutrophils undergo a respiratory burst which produces large quantities of the reactive oxygen species which are generated by the activation of NADPH oxidase. Neutrophil granules containing myeloperoxidase catalyze the formation of hypochlorous acid through the reaction of hydrogen peroxide with chloride (Kruger et al. 2015).

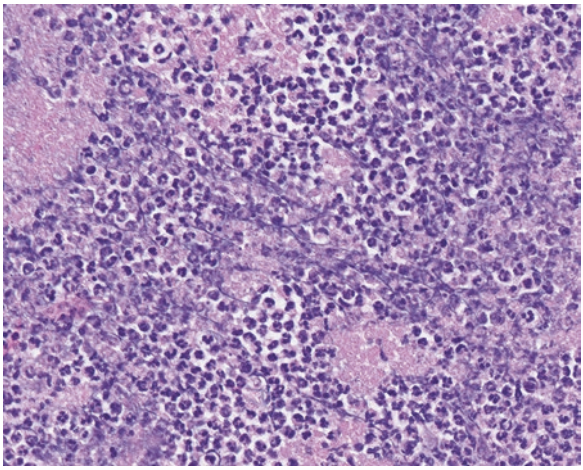

Fig. 1.4 A subcutaneous abscess in a mouse has numerous partially degenerated neutrophils and pools of eosinophilic (*pink-stained*) debris. Note the strands of basophilic (*blue*) material, which have traditionally been considered to represent nucleoprotein debris from degenerated neutrophils. Current evidence suggests these fibrinous strands may contain functional Neutrophil Extracellular Traps (NETs). NETs are composed of neutrophil-origin nucleic acids and serine proteases that serve to trap and kill bacteria. Similar extracellular traps are generated by eosinophils, basophils and mast cells. H&E stain, 40× objective magnification

Additionally, the granules of neutrophils contain a variety of compounds such as defensins, myeloperoxidase, serine proteases, neutrophil elastase, collagenase, cathelicidin, and gelatinase, which all can contribute to the antimicrobial action. NETs are comprised of a web of chromatin fibers and serine proteases that form a physical barrier that helps to trap and kill microbes (Brinkmann et al. 2004; Fig. 1.4). Neutrophils are also professional phagocytes which rapidly phagocytotize pathogens, particularly those coated with complement or antibodies, and damaged cells or debris. The role of neutrophils in modulating immune cells is less characterized but distinct neutrophil phenotypes exist which, rather than activating immune responses, may inhibit T and NK cell proliferation. This population of neutrophils is known as granulocytic or neutrophilic myeloid-derived suppressor cells (MDSC) (Mayadas et al. 2014; Kruger et al. 2015). Additionally, neutrophils have been shown to influence with other immune cells including DCs, macrophages, lymphocytes and NK cells, as well as non-immune cells including endothelial and epithelial cells (Kruger et al. 2015; Mayadas et al. 2014; Nathan 2006). Once thought of as simple immune cells with a single focus of toxicity, the heterogeneity and plasticity of neutrophils is increasingly being recognized and subpopulations based on function and/or phenotype have been described (Kruger et al. 2015).

Eosinophils have granules which stain eosinophilic (i.e., pink to red) with traditional cytologic and histologic stains. These cells play an important role in both protecting against helminth pathogens and also in the pathogenesis of allergies and asthma. Eosinophils are primed for activation by IL-3, IL-5 and GM-CSF and can

be activated by a variety of mechanisms including cross-linking of IgA or IgG Fc receptors (Stone et al. 2010). Following activation, eosinophil degranulation can occur via exocytosis and cytolysis. The granules within eosinophils contain cytotoxic substances, the primary ones being major basic protein (MBP) and cathepsin (a cationic protein), both of which are toxic to parasites. MBP's toxicity is thought to be mediated by enhancing membrane permeability of invading pathogens (e.g., helminths). Additional products, such as, eosinophil-derived neurotoxin and eosinophilic cationic protein (ECP) are toxic to parasites and ssRNA pneumovirus while eosinophil peroxidase (EPO) produces oxidant products which are toxic to microbes and host cells alike (Stone et al. 2010). Eosinophils also produce LTC4, PGE2, thromboxane, platelet-activating factor and an array of cytokines). Eosinophils enhance early B cell activation in T cell-dependent immune responses and have receptors which bind to IgE, which mediates much of their role in the pathogenesis of respiratory system diseases (Berek 2016; Eng and DeFelice 2016). There is recent evidence that eosinophils play an important role in promoting long-term survival of plasma cells in the bone marrow and lamina propria of the gastrointestinal system, and are necessary for supporting efficient class-switching of B cells to IgA and generation of IgA (Berek 2016; Travers and Rothenberg 2015).

Basophils, the least abundant circulating granulocyte population, have granules which stain basophilic (i.e. blue) with traditional cytologic and histologic stains. Basophils play important roles in inflammatory, allergic and parasitic infections, and have additional roles in humoral adaptive immune responses (Siracusa et al. 2011; Stone et al. 2010). Basophils are differentiated primarily under the influence of IL-3 (Stone et al. 2010). The granules of basophils have abundant histamine, heparin, peroxidase and numerous other compounds. Histamine causes dilation and increased permeability of capillaries which facilitate and enhance inflammatory responses. Basophils bind IgE via FcεR1 and, upon activation, release histamine. Although originally considered a redundant mast cell-like population, they differ in various ways (Siracusa et al. 2011). Basophils also rapidly produce large amounts IL-4 and IL-13 which contributes to their role in promoting T_H2 responses (Stone et al. 2010). In addition to FcεR1 activation, basophils can also be activated by complement components C3a and C5a, TLR2 and TL4, numerous cytokines (e.g., IL-4, IL-13, GM-CSF), proteases and antibodies, as well, as preformed mediators including histamines, LTC4 and anti-microbial peptides) (Siracusa et al. 2011). Basophils have also been implicated as possible antigen-presenting cells, and support antigen-specific T_H2 responses in the context of allergen or helminth exposure (Perrigoue et al. 2009; Siracusa et al. 2011; Sokol et al. 2009; Yoshimoto et al. 2009).

Mast cells are tissue-based immune cells that play central roles in the pathogenesis of immediate hypersensitivities, but also play roles in host responses to various disease states (e.g., pathogens, fibrosis, autoimmunity and wound healing). Mast cells have granules which contain histamine, serine proteases (tryptase and chymase) , carboxypeptidase A, proteoglycans and neutral proteases (Stone et al. 2010). Mast cells function in homeostasis to promote innate and adaptive immune responses and wound healing. Mast cells are activated through FcεRα whereby bound IgE leads to release of histamine. It is important to recognize there are two distinct

populations of tissue mast cells. The widely distributed type of mast cells, also known as connective tissue mast cells, are readily visualized in the H&E tissue stains that are commonly used in the histopathology evaluation of toxicology studies. A second population of mast cells, known as mucosal mast cells, is limited to mucosal surfaces such as the gastrointestinal and respiratory tracts. The cytoplasmic granules of mucosal mast cells are not discernible in H&E-stained sections unless the tissues are fixed in special fixatives, thus the mucosal mast cell population is not easily detected. This feature of mast cells becomes very important in situations where xenobiotic-mediated gastrointestinal or respiratory sensitization is suspected (Wingren and Enerback 1983; Enerback 1987; Strobel et al. 1981).

Natural killer (NK) cells and NK-T cells: Natural killer cells, an innate immune lymphoid cell type that is present throughout the body, plays an important role in mediated cellular killing of target cells, in some ways similar to $CD8^+$ cytotoxic T cells (Sun and Lanier 2011; Bjorkstrom et al. 2016). Their major function is to kill infected cells, which is accomplished primarily do through exocytosis of proteins within granules. Perforin is a granule protein which facilitates the entry of other proteins (i.e., granzymes) into the cytoplasm of the target cell, inducing a series of events in the target cell that ultimately result in apoptosis of the target cells (Abbas and Lichtman 2015). NK cells also respond to IL-12 and are potent producers of IFN-γ which further activates macrophages to destroy phagocytosed microbes (Abbas and Lichtman 2015). In addition to their roles in targeting virally infected cells and enhancing macrophage killing of intracellular microbes, NK cells also play an important role in recognizing healthy cells and eliminating unhealthy or stressed cells. NK cells recognize MHC class I expression when it is appropriately expressed on host cells. If there is inhibition or alteration of MHC class I expression such as during viral infection or in a cancer cell, NK cells will lyse the target cell.

Killer cell immunoglobulin (Ig)-like receptors (KIRs) are activating and inhibitory receptors which recognize numerous ligands that are expressed primarily on cells that have been infected with a microbe, are transformed or have undergone stress (Abbas and Lichtman 2015). NKG2D is an activating KIR that is found on virally-infected cells and tumor cells but not on normal cells. NKG2D association with its signaling subunit DAP10 enhances NK cell cytotoxicity. CD16, a phenotypic receptor present on numerous NK cells, is FcγRIIIA, which is a low-affinity receptor for IgG antibodies. When IgG antibodies bind to microbial antigens expressed on the surface of an infected cell, CD16 on NK cells binds these antibodies and kills the infected cell through a process called antibody-dependent cell-mediated cytotoxicity (ADCC). To avoid unregulated cellular killing, NK cells have inhibitory receptors that recognize MHC class I molecules. These include inhibitor KIRs, lectins, such as CD94/NKG2A heterodimer and leukocyte Ig-like receptors (LIRs), all of which recognize various MHC class I molecules and inhibit the cellular killing activities of NK cells. Emerging receptors are being identified, and a family of molecules has recently been described that recognizes nectin and nectin-like proteins (Martinet and Smyth 2015). Downstream signaling events include immunoreceptor tyrosine-based activation motifs and immunoreceptor tyrosine-based inhibition motifs (ITAMs and ITIMs, respectively), and it is the ultimate inte-

gration of activating and inhibitory receptor signaling which determines the outcome of NK cell interaction with other cells.

NK-T cells are a unique T cell subset that share properties with and phenotypic markers of both T cells and NK cells. Many recognize the non-polymorphic CD1d molecule (i.e., are CD1d-restricted). CD1, a member of the family of MHC-like antigen-presenting molecules, binds self and foreign lipids and glycolipids. There are several subtypes of NK-T cells, including type 1 (invariant), type 2 and NKT-like cells which have relatively distinct functions including the rapid release of cytokines and chemokines including IFN-γ, IL-4, TNF-α, GM-CSF, IL-2 and IL-4, depending on the NK-T population involved and the nature of stimuli (Godfrey et al. 2004; Abbas and Lichtman 2015).

Macrophages are myelomonocytic cells that have primary functions in both the innate and adaptive immune systems and, like DCs, are professional APCs which can influence adaptive immune responses. Macrophages can be tissue-resident or arise from monocytes following recruitment to sites of inflammation or need. Macrophages are widely distributed throughout the mammalian body and have myriad functions, including phagocytic clearance of pathogens and cell debris, production of cytokines, chemokines and eicosanoids, and induction of multiple modulations of other lymphoid cells. Recent data redefine macrophages as diverse polyfunctional cells that exhibit substantial plasticity in response to the needs of local tissues during steady state homeostasis and pathologic processes. Macrophages not only modify their phenotype and function in response to signals received from local tissues, but also undergo dynamic and continuous adaptation in response to changing needs of the local tissue. Macrophages were historically thought to be uniformly derived from circulating monocytes that originate in the bone marrow. It now known there is a separate population of 'tissue resident' macrophages that originate from mesenchymal elements during three successive and somewhat overlapping periods in the fetus, and co-exist with hematopoietic-origin (bone marrow-derived) macrophages in the adult host. The population of tissue-resident macrophages includes microglial cells of the brain, Langerhans cells of the skin, Kupffer cells of the liver, and at least a subpopulation of alveolar macrophages of the lung. In these locations the tissue-resident macrophages serve as accessory cells, performing tasks such as removal of apoptotic bodies, thus freeing cells of the local tissue for participation in their highly specialized functions. The final phenotype and function of macrophages is determined by the lineage hierarchy, signals continually received from the tissue of residence, and additional signals related to immediate demands.

Macrophages are primary effector cells of both the innate and adaptive immune systems, and have highly specialized functions that are served by specific adaptations of the macrophages as well as precise locations within the lymphoid tissues. For example, macrophages in different regions of lymphoid tissues may be dedicated to either antigen presentation or phagocytic clearance of particulate material, including cell debris (den Haan and Martinez-Pomares 2013). In secondary lymphoid tissues such as the spleen and lymph nodes, sites of incoming antigen exposure have significant populations of $CD169^{+}$ macrophages (den Haan and

Martinez-Pomares 2013). These CD169^{high} macrophages are located in the subcapsular sinuses of lymph nodes and marginal zones of the spleen ('marginal metallophilic macrophages'), where their proximity to B cell follicles promotes immune recognition, while CD169^{low} macrophages are located in the red pulp and outer marginal zone of the spleen and medulla of lymph nodes, where they are involved in clearance of particles and molecules. The phagocytic function of CD169^{low} macrophages in the spleen is of particular interest to toxicologists and toxicologic pathologists because of the role these macrophages have in clearing effete erythrocytes from the circulation. When test articles with oxidizing properties bind to erythrocytes, the deformability of the erythrocytes may be reduced. Splenic macrophages rely to some extent on this characteristic of erythrocytes to determine whether individual erythrocytes should be removed from the circulation. Thus test article-related oxidation of erythrocyte membranes may result in hematologic evidence of anemia and possible adverse effects on the spleen due to the high dose of the article that is captured by macrophages in the spleen. Macrophages not only play important direct immune roles but also contribute to positive and negative selection processes in the thymus and mobilization of hematopoietic stem cells in the bone marrow and play important roles in the thymus during positive and negative selection processes (Winkler et al. 2010; Chow et al. 2011; Ehninger and Trumpp 2011; Ludin et al. 2012; Surh and Sprent 1994; Ravichandran 2011; Segawa et al. 2014).

Macrophages are markedly heterogeneous in their phenotype and function states (Gordon and Taylor 2005). The local tissue and microenvironment can influence macrophage activation state and promote polarization into classically activated (i.e., M1) or alternatively activated (M2) macrophages (Martinez and Gordon 2014; Gordon and Martinez 2010). Classically activated macrophages have enhanced antigen-dependent enhanced microbicidal macrophage reactivity that is associated with T_H1 responses and IFNγ production by activated T cells, and to cytotoxic and antitumor properties (Nathan et al. 1983; Pace et al. 1983; Celada et al. 1984; Mackaness 1962). These classically activated macrophages play an important role in enhancing cell-mediated immune responses important for combatting intracellular bacteria or viruses. Following the discovery of classically activated macrophages, it was subsequently discovered that the mannose receptor on macrophages was specifically enhanced by IL-4 and IL-13, which are major signals of the T_H2 response. Enhanced activity of the mannose receptor in macrophages caused an increased rate of endocytic clearance of manosylated molecules, increased expression of MHC class II, and reduced production of pro-inflammatory cytokines. The mannose-based activation pathway was distinctly different from the classical pathway, resulting in macrophages that were not 'activated' in the classical sense, but certainly not deactivated (Stein et al. 1992; Doyle et al. 1994). The classical and alternative pathways of macrophage activation have been termed M1 and M2, in part to match the dichotomous symmetry between T_H1 versus T_H2 responses (Mills et al. 2000). The M1/M2 paradigm was later subdivided into M1 (IFNγ + LPS or TNF), M2a (IL-4), M2b (immune complex + TLR ligands), and M2c (IL-10 and glucocorticoids). The M1/M2 paradigm retains some utility when considering the immunologic reactions of macrophages, but current data suggest classification of

macrophages and their functions is much more complicated than suggested by the M1/M2 classification (Martinez and Gordon 2014). See (Martinez and Gordon 2014) for more details on the macrophage M1/M2 paradigm. The development of M1 versus M2 responses can have dramatic impacts on subsequent downstream immune responses. For that reason, there is increasing interest in targeting innate immune cells such as the macrophage with immunotherapies to influence the overall nature of macrophage and downstream immune functions (Pei and Yeo 2015; Talekar et al. 2015).

Dendritic cells (DCs) are myelomonocytic cells which arise from hematopoietic precursors and commonly reside at sites of pathogen interface (e.g., skin and mucosal sites) and within immune organs to facilitate innate-adaptive immune interactions (Steinman 2006). Similar to macrophages, dendritic cells are present in multiple tissues, although they typically do not have unique organ-specific names (Malissen et al. 2014). Both conventional DCs and plasmacytoid DCs as well as subsets within these populations have been described (Kushwah and Hu 2011). Conventional DCs have functions that are similar to macrophages in promoting specific adaptive immune response, while plasmacytoid DCs play an important role in anti-viral defense by producing abundant IFN-α (Abbas and Lichtman 2015). A detailed discussion of pDCs is beyond the scope of this chapter but reviews are available (Lande and Gilliet 2010; Villadangos and Young 2008; Kaisho 2010).

The remainder of this discussion will focus on the nature and function of conventional DCs. Immature DCs are highly endocytic, expressing low levels of activation markers which limit their ability to promote T cell activation. These DCs survey the environment via phagocytosis and, following exposure to antigens and environmental stimuli, such as PRRs and TLRs, become mature DCs. These DCs then commonly migrate to secondary immune organs (i.e., lymph nodes) where they can initiate immune responses, activate T cells and influence the development of cell-mediated or humoral adaptive immune responses. DCs process antigens and typically present them in an MCH II-restricted manner. Although macrophages and B cells are also professional APCs, DCs are considered unique in their ability to induce differentiation of naïve T cells. DCs also play an important role in the initiation and maintenance of tolerance. DCs are highly potent immune system cells that are increasingly considered as a therapeutic target, both for delivery of a therapeutic payload or as a cell-based therapy (Ahmed and Bae 2014; Klechevsky and Banchereau 2013; Palucka and Banchereau 2013; Steinman and Banchereau 2007).

MDSCs: Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cell precursors that are commonly increased in number in pathologic states. MDSCs were first described for their role in suppressing immunity in cancer, but there is additional evidence they play important regulatory roles in other conditions and disease states (Gabrilovich and Nagaraj 2009; Serafini et al. 2006). MDSCs are precursor cells in normal hematopoiesis that have the potential to give rise to monocytes/dendritic cells or granulocytes such as neutrophils. These MDSC subpopulations are divided into either granulocytic or monocytic based largely on cell surface marker expression consistent with these specific populations,

as well as functional attributes more aligned with one versus the other mature cell population (Gabrilovich and Nagaraj 2009). However, numerous additional markers and functional attributes have been discovered and attributed to various MDSC populations depending on the species and disease state/experimental system evaluated. MDSCs can modulate ("suppress") immune responses through a variety of mechanisms including arginase, nitric oxide, prostaglandin E2, IL-10 and other cytokine expression which directly induce regulatory effects or promote the production of regulatory responses in both innate and adaptive immune cells (Gabrilovich and Nagaraj 2009; Nagaraj et al. 2013; Ostrand-Rosenberg et al. 2012). For example, MSDCs are associated with increased arginase and iNOS levels, both of which inhibit T cell function and/or proliferation, and increased production of reactive oxygen species (ROS) such as nitric oxide (NO) and peroxynitrite, both of which suppress T cell function. (Peroxynitrite nitrates the T cell receptor (TCR) and CD8 molecules, resulting in altered peptide binding to the TCR which renders T cells unresponsive to antigen-specific stimulation (Gabrilovich and Nagaraj 2009; Bronte and Zanovello 2005; Rodriguez and Ochoa 2008). MDSCs are also known to induce regulatory T cells by several mechanisms, including CTLA-4 expression by MDSCs, arginase-dependent mechanisms, and altered costimulatory molecule expression (Gabrilovich and Nagaraj 2009). Although a relatively recent addition to the population of immune cells, MDSCs are important regulators of both innate and adaptive immunity during health and disease, and are important cells to consider in developing immunotherapies (Seledtsov et al. 2015; Ugel et al. 2009).

1.3.5 Antigen Processing and Presentation

Major histocompatibility complex (MHC) class I and class II molecules present antigenic peptides on cell surfaces to $CD8^+$ and $CD4^+$ T cells, respectively. Each type of APC interacts with T cells that express T cell receptor (TCR), but the class of MHC molecule associated with the TCR determines the T_H cell helper pathway that is activated (Trombetta and Mellman 2005). Different intracellular pathways and mechanisms are involved in the degradation and processing of the endogenous versus exogenous proteins that ultimately result in antigenic peptides (Vyas et al. 2008). MHC class I presents antigens derived from the 'cytosolic pathway', i.e., peptides derived from cytoplasmic self-proteins and intracytoplasmic pathogens, which stimulates the differentiation of $CD8^+$ T cells into cytotoxic T cells. MHC class II presents antigens derived from the 'endocytic pathway', i.e., peptides that result from endosomal/lysosomal degradation of self- and non-self proteins, which serves to activate $CD4^+$ T_H cells. There are exceptions to these general rules, as exogenous peptides can be presented by MHC class I through the process of cross-presentation, and endogenous peptides can be presented by MHC class II if the endogenous antigenic peptides are derived via the intracellular process of autophagy rather than transmembrane acquisition of peptides via phagocytosis or pinocytosis (Crotzer and Blum 2010).

MHC-I and MHC-II are the classic human MHC molecules. MHC-III loci map to the MHC region, but code for complement proteins. MHC terminology varies with different species. MHC genetic loci are indicated by designations in *italics*, and the associated proteins are shown in standard font. Human = *HLA* (for 'human leukocyte antigen'), Mouse = *H2,* Rat = *RT1,* Dog = *DLA,* Guinea pig = *GPLA,* Swine = *SLA,* and Rabbit = *RLA (Margulies et al.* 2013*)*. Klein proposed using the first two letters of genus and species names for MHC molecules in nonhuman primates. E.g., *Mafa* for cynomolgus monkey (*Macaca fascicularis*) or *Mamu* for rhesus monkey (*Macaca mulatta*) (Klein 1986).

1.3.5.1 MHC Class I

MHC class I is expressed by all nucleated mammalian cells, and presents antigenic peptides derived from cytosolic or nuclear proteins via proteasomal digestion. Peptide fragments in the proteasome are transported to the endoplasmic reticulum (ER) by transporters of antigenic peptides (TAPs), where the antigenic peptides encounter newly formed MHC class I molecules. The antigenic peptides, typically consisting of 8 and 9 amino acids, are inserted into a deep groove in the MHC class I complex that consists of a polymorphic alpha chain and a β_2 microglobulin molecule. Presence of the peptide in the MHC class I complex stabilizes the complex, allowing surface expression of the antigenic peptide. Because of the inherent instability and rapid degradation of many newly formed cytosolic protein, many MHC class I molecules do not receive the antigenic peptide fragment that conveys stability to the overall molecular complex, and are degraded by the ER-associated protein degradation (ERAD) system (Hughes et al. 1997).

The proteasome is a functional unit that is involved in degradation of cytosolic proteins, typically effete proteins that have reached the end of their effective lifespan. The prototypic proteasome (the 26S proteasome) consists of a 20S core barrel with protease activity and two 19S caps. Alternate forms of proteasomes include the immunoproteasome, which is present in multiple immune system cells, and thymus-specific proteasomes, which are present in thymic epithelial cells (Sijts and Kloetzel 2011). Immunoproteasomes generate peptide fragments that subsequently associate with the various subgroups of MHC class I molecules (Neefjes et al. 2011).

While MHC class I molecules are involved in presentation of peptides derived from effete intracellular proteins, these effete proteins are not the only source of peptides for expression by MHC class I. It is estimated that 30–70% of all synthesized proteins are defective for various reasons, thus are known as defective ribosomal products (DRiPs) (Li et al. 2011; Yewdell and Hickman 2007; Berglund et al. 2007; Dolan et al. 2011; Netzer et al. 2009). The DRiPs are immediately degraded and associated antigenic peptides are expressed in context of MHC class I molecules. As a result of this process, DRiPs associated with viral infection may be expressed by MHC class I several hours before stable viral proteins are expressed, thus allowing T cells to mount an early response to infections such as influenza virus infection (Khan et al. 2001).

MHC class I molecules are not entirely limited to presentation of peptides that originated from the individual cell that bears the MHC molecule. Many cells are linked to adjacent cells by gap junctions, and peptides can move between cells via these junctions. Gap junctions remain functional in the early stages of apoptosis, thus potentially antigenic peptides contained within a dying cell can move into adjacent viable cells and be presented by MHC class I molecules in the destination cell (Pang et al. 2009). Dendritic cells in lymph nodes form gap junctions with nearby cells, thus promoting cross-priming via gap junction transfer of antigenic peptides. Gap junction transfer of antigenic peptides from neoplastic cells to APCs can promote anti-tumor immunity (Saccheri et al. 2010).

The number of cytosolic peptide fragments resulting from proteasomal degradation far surpasses the number of MHC class I molecules, which suggests the peptide-binding capacity of the MHC class I molecules should be perpetually overwhelmed. However, as presented above, many MHC class I molecules do not receive a stabilizing antigenic. This apparent paradox is explained by the fact that the peptides have a half-life of only 6–10 s. Those few peptides that encounter transporters of antigenic peptide (TAP) carrier molecules and MHC class I molecules during their brief existence are eventually presented as antigenic peptides on the cell surface. The great majority of the remaining peptides are degraded by aminopeptidases in the cytosol and, as presented above, the MHC class I molecules that do not receive antigenic peptides are degraded by the ERAD system. As a result, only a sampling of the cytosolic proteins is eventually presented to T cells in MHC class I context.

As MHC class I molecules reach the end of their functional lifespan, they are internalized by phagocytosis and proceed through the endosomal/lysosomal degradative pathway. Acidification of the MHC class I/peptide complex in lysosomes results in the release of the antigenic peptides, which can subsequently be incorporated into MHC class II molecules and presented to a different subset of T cells.

1.3.5.2 MHC Class II

MHC class II molecules are expressed primarily by professional antigen-presenting cells (APCs) such as dendritic cells, macrophages and B cells, though under some circumstances MHC class II is expressed in non-APCs such as mesenchymal stromal cells (Romieu-Mourez et al. 2007), fibroblasts and endothelial cells (Geppert and Lipsky 1985), and a variety of cells involved in pathologic processes such as Crohn's disease (Bland 1988) and psoriasis (Schonefuss et al. 2010). B cells lack the endosomal/lysosomal compartment that is present in macrophages. To compensate for this deficiency, B cells produce a modifier protein that associates with MHC class II molecules and restricts activity to acidic compartments similar to the lysosomal compartments of macrophages (Denzin et al. 2005). Thus, in all the major categories of MHC class II-expressing cells, the antigenic peptides are largely formed by acidic digestion of exogenous proteins.

Different types of antigen-presenting cells (APCs) acquire antigen by different pathways. Dendritic cells (DCs) sample their environment and capture multiple

foreign antigens such as those derived from pathogens, and present those antigens in the context of MHC for activation of $CD4^+$ and $CD8^+$ T cells (Banchereau et al. 2000). Epithelial cells and DCs of the thymus express and capture numerous self-proteins that are used in the generation of central and peripheral tolerance. In contrast to the pleuripotent antigen presentation of the 'professional' APCs, B cells typically capture only a single antigen that is bound to an antigen-specific receptor on the surface of the B cell (Harwood and Batista 2010).

The α and β chains of MHC class II are produced in the endoplasmic reticulum of APCs, where they associate with the invariant chain (Cresswell 1996) before passing through the Golgi apparatus to their final destination in the clathrin-coated pits of the plasma membrane (McCormick et al. 2005; Dugast et al. 2005) The invariant chain-MHC complex is internalized into endosomes along with phagocytized proteins (Busch et al. 2005), and subsequently passes into the phagolysosome ('antigen-processing compartment') of the APC. Up to this stage it is not possible for antigenic peptides to associate with the nascent MHC class II molecule due to the presence of invariant chain in the peptide-binding groove of the MHC molecule (Roche and Cresswell 1990). Subsequent partial proteolysis of the invariant chain results in the formation of class II-associated invariant chain peptide (CLIP), which remains in the peptide-binding groove of the MHC molecule. CLIP is replaced by antigenic peptides derived from proteolysis in the antigen-processing compartment (Busch et al. 2005; Denzin et al. 2005), thus forming the final MHC class II molecule containing antigenic peptide. Transport vesicles bearing the completed MHC class II molecules with antigens fuse with the cell membrane, resulting in the cell surface expression of antigenic peptide contained within the antigen-presenting groove of MHC class II molecules (Roche and Furuta 2015).

In addition to presentation on the surface of cells, fragments of APC membrane bearing antigen-loaded MHC class II molecules may be released as exosomes into the extracellular space. Integration of the exosomes laden with MHC class II/antigenic peptide into recipient cells can result in 'cross-dressing', i.e., antigen presentation in MHC class II context by cells that do not normally express MHC class II.

Antigenic peptides enter the endocytic pathway by multiple mechanisms. Clathrin-mediated endocytosis, macropinocytosis, and phagocytosis internalize proteins into endosomes, macropinosomes or phagosomes, respectively (Lim and Gleeson 2011; Clement et al. 2011; Rock et al. 1984; Stuart and Ezekowitz 2005). Macropinocytosis is particularly important in acquisition of antigens by dendritic cells, which exhibit a transient burst of macropinocytosis upon activation (West et al. 2004). The protein-laden structures that result from the various endocytotic pathways then fuse with the antigen-processing compartment, followed by proteolytic release of peptides that follow the above pathway for incorporation into MHC class II surface expression. Antigenic peptides are also derived from internal cellular structures through the process of autophagy (Adamopoulou et al. 2013; Crotzer and Blum 2010; Schmid et al. 2007; Deretic 2011), which has subcategories of macroautophagy, microautophagy and chaperone-mediated autophagy (Levine and Deretic 2007). In macroautophagy, an autophagosome is formed around effete cytoplasmic structures, using membrane components that are largely derived from the endoplas-

mic reticulum. In microautophagy, effete cytoplasmic elements bud into pre-existing lysosomes, resulting in membrane-bound intra-lysosomal bodies. In chaperone-mediated autophagy, cytoplasmic elements bind to specific receptors on the surface of lysosomes, which are subsequently internalized into the lysosomes. Thereafter the fate of proteins internalized via autophagy is similar to that of proteins internalized by other pathways, with the final result of antigenic peptides expressed in the context of MHC class II molecules on the cell surface. The process of chaperone-mediated autophagy is specific for certain proteins, thus the process may be amenable to pharmacological intervention and alteration of immune-mediated processes which are initiated by this mechanism of antigen presentation to T cells.

See (Roche and Furuta 2015) for a review of MHC class II antigen processing and presentation. See (Deretic 2011; Levine et al. 2011) for reviews of autophagy involvement in immunity and inflammation.

1.4 Adaptive Immune System Overview

In contrast to the innate immune response, the adaptive immune response (also called specific or acquired immunity) is able to respond in a specific manner dependent upon the insult (e.g., infectious agent) and the associated antigens presenting by APCs. The two broad arms of the adaptive immune response are the cellular and humoral responses. The cellular immune response is effected by cellular constituents such as lymphocytes, while the humoral arm is effected by secreted products (antibodies). Cell-mediated immune responses primarily target intracellular microbes which include both viruses, as well as, intracellular bacteria. Both cytotoxic T cells and helper T cells help promote functional abilities (e.g., intracellular killing abilities of infected macrophages and overt killing of infected cells. Humoral immune responses are primarily employed to remove extracellular microbes. Both B cells that produce antibodies and helper T cells which help promote humoral immune responses (e.g., by promoting B cell differentiation into plasma cells, production of T_H2 cytokines, etc.) are involved in the process of preventing or eliminating these organisms. However, dysregulated or excessive humoral immune responses can also contribute to pathologic states such as allergy, asthma and antibody-mediated autoimmune diseases.

The specific and amnestic responses that characteristize the adaptive immune response are accomplished by T and B lymphocytes, which are derived from multipotent hematopoietic stem cells. T cell progenitors migrate from the bone marrow to the thymus where they develop into T cells. Through a process of negative and positive selection, populations of helper $CD4^+$ and cytotoxic $CD8^+$ T cells are produced. B cell progenitors are present in the bone marrow in mammals (or bursa of Fabricius in avian species) and can become plasma cells following activation in peripheral lymphoid tissues. B cells can also serve as professional APCs. An overview of lymphocyte function is presented below, and Volume 1, Chap. 2 highlights the development and cellular features of lymphocytes.

1.4.1 CD4+ T Cell Activation and Function

Naïve helper T cells differentiate into a variety of subpopulations under the influence of various transcription factors, and then have well-defined functional characteristics. This single-fate model for specific T cell subpopulations is useful in understanding T cell functionality. However, it is increasingly recognized there is a significant degree of flexibility, plasticity and cross-talk between T cell populations and their ultimate destiny (Coomes et al. 2013; Tripathi and Lahesmaa 2014). For the sake of clarity, we will focus on some of the key helper T cell subsets, factors which influence and promote their differentiation, and the relative function of each of these subsets (Tripathi and Lahesmaa 2014; Coomes et al. 2013; Zhu et al. 2010).

$CD4^+$ effector T cells play important roles in defense against pathogens and maintaining homeostasis by helping to activate and recruit phagocytes and other immune cells as well as helping B cells produce and refine their production of antibodies (Abbas and Lichtman 2015). Naïve helper T cell differentiation relies on antigen presentation by DCs while macrophages and B cells can serve APC functions for cell-mediated immunity and humoral immunity effector T cell responses, respectively (Abbas and Lichtman 2015). In certain circumstances, additional cells such as endothelial cells and various epithelial and mesenchymal cells can serve as non-professional APCs. Signals from the environment which stimulate or activate (e.g., microbial products, adjuvants, etc.) promote the activation of APCs which results in up-regulation of costimulatory molecules (i.e., Signal 2), which is critical to ensure appropriate immune responses rather than a tolerogenic response.

Naïve helper T cells (a.k.a. Th_0 cells) are activated following recognition of a peptide-MHC class II complex presented on dendritic cells (Fig. 1.5). T cells and their receptors have a pre-defined specificity determined during development during the process of V(D)J rearrangement. T cell receptors (TCR) recognize the peptide-MCH complex (Signal 1) which, in the presence of appropriate co-stimulation (Signal 2) and influenced by the local microenvironment (Signal 3), results in differentiation of T cells. It is the effective orchestration of these signals which influences the balance between effective immunity and tolerance (Abbas and Lichtman 2015; Cools et al. 2007; Zhu et al. 2010; O'Shea 2013). This first step in T cell polarization (or differentiation) of helper T cell subsets from naïve $CD4^+$ T cells is termed the induction phase. The commitment phase is the next phase where epigenetic modification and continued activation progressively promotes the commitment to one specific pathway of differentiation. Amplification, the third stage, results from specific microenvironmental cues and results in an expanded population of cells of a particular subset.

Signal 1: Antigen-presentation by an APC to a TCR is the 1st signal. Resting (immature) tissue-resident DCs are the APCs which are critical for influencing the differentiation of naïve helper T cells. These immature DCs, upon capturing antigen, migrate via lymphatics, become activated and present antigen to naïve T cells in local draining lymphoid organs (i.e., lymph nodes). Extracellular proteins (exogenous) are taken up and processed by APCs into peptide fragments which are

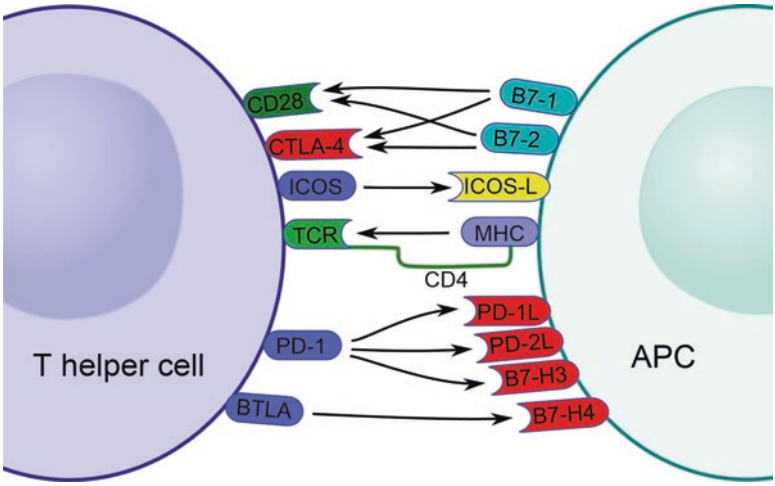

Fig. 1.5 Helper T cell activation. Activation of helper T cells by antigen-presenting cells (APC) involves multiple signaling pathways between helper T cell and APC. These molecules and signaling pathways provide numerous potential targets for pharmacological intervention. See text Sect. 1.4.1 and associated references for additional details

loaded internally onto the peptide-binding cleft of MHC class II molecules. The peptide-MHC complexes are then presented on the surface of APCs, where they interact with naïve $CD4^+$ helper T cells that have specificity for the peptide-MCH complex. The TCR is part of a larger complex composed of the TCR αβ heterodimer non-covalently associated with signaling molecules such as CD3 and ζ proteins. Following the formation of the MHC/antigenic peptide/CD3 trimer on the cell surface, CD3 and ζ proteins transduce intracellular signals that result in T cell activation. It is the recognition of the MHC-associated peptides recognized by the TCR complex that triggers the formation of an immunological synapse which is the clustering of various co-receptors, signaling molecules and cell surface proteins necessary to form a stable contact between the T cell and APC. The formation of the immunological synapse allows for assembly of signaling components including costimulatory receptors, adaptors and co-receptors with the necessary temporal and spatial interactions to facilitate appropriate signaling. Over time, the TCR signaling eventually is inhibited via tyrosine phosphatases which remove phosphate moieties (e.g., SH2 domain-containing phosphatases 1 and 2; SHP-1 and SHP2), signal molecule degradation by E3 ubiquitin ligases or other mechanisms (Abbas and Lichtman 2015).

Signal 2: Co-stimulation is the 2nd signal for T cell activation. The most characterized and utilized co-stimulatory interactions are those of the B7:CD28 families. Microbial products, adjuvants, engagement of TLRs and various cytokines can influence the expression patterns of co-stimulatory molecules on APCs and T cells. Resting or unactivated APCs typically present self-antigens to T cells in the presence of low levels of co-stimulation, which contributes to the formation of tolerogenic

cells. When Signal 1 occurs in the absence of Signal 2, T cells become unresponsive or tolerant or may die by apoptosis (Abbas and Lichtman 2015). Alternatively, marked and prolonged signaling in the presence of high antigen and co-stimulatory levels can result in deletion of reactive T cells. These are types of peripheral tolerance that arise during the process of normal immune responses whereby absent or variable levels of Signals 1 or 2 result in tolerant cells. This form of tolerance is in contrast to the central tolerance which occurs in the thymus during negative selection.

APCs up-regulate the expression of co-stimulatory molecules such as CD80 and CD86 (B7-1 and B7-2, respectively) following activation or stimulation by microbial agents or other stimuli. CD80 and CD86 are members of the B7 superfamily which is comprised of numerous members which can be both stimulatory and inhibitory in nature (Sharpe and Freeman 2002).

When the expression of co-stimulatory molecules is upregulated, CD28 present on the surface of T cells can bind to the co-stimulatory molecules and provide an effective 2nd signal to promote T cell differentiation. Depending upon the combinations of co-stimulatory molecules expressed and their respective ligands, various cell-mediated and humoral immune responses can be generated. Inducible costimulator (ICOS) and its ligand are particularly important in T cell-dependent antibody responses and in the germinal center reaction. CTLA-4 is a co-stimulatory molecule whose primary purpose is to regulate and dampen T cell activation and proliferation. T cells typically have low levels of CTLA-4. However, upon activation, CTLA-4 is expressed and limits the proliferation and expansion of responding T cells, thus applying a brake to an activation process that could be unending and ultimately damaging to the host. CTLA-4 present on Tregs inhibits the activation of T_H cells by binding to B7 molecules (CD80 and CD86) on the surface of APCs, which prevents the binding to the activating co-stimulatory CD28 molecules. The inhibitory functionality of CLTA-4 and several other inhibitory co-stimulatory molecules, known as checkpoint blockade, has been investigated or developed for therapeutic applications. Some examples include PD-1 (programmed cell death-1) and its ligands PD-L1 and PD-L2. OX40 is another co-stimulatory molecule which is expressed on $CD4^+$ and $CD8^+$ T cells, and functions to maintain cell survival and sustained responses. There are also receptor-ligand interactions which can amplify T cell responses not by directly serving as a co-stimulatory molecules for T cells, but rather by inducing co-stimulatory molecule expression on APCs. CD40 ligand on activated T cells binds CD40 on APCs, which activates macrophages (for cell-mediated immune responses) and B cells (for humoral immune responses), promotes up-regulation of co-stimulatory molecules like CD80/CD86, and stimulates cytokine secretion, all of which amplify T cell responses.

Signal 3: In addition to the critical signals of MHC-peptide-TCR and co-stimulatory molecule influences, the local microenvironment can have a large impact on the differentiation of specific T cell populations. The composition of cytokines and chemokines is extremely important in driving the differentiation of various helper T cell subsets and there is abundant cross-talk, regulation and counter-regulation between these cytokines that will be reviewed in detail later in

this chapter. In addition to the influence of cytokines and chemokines, the local microenvironment, tissue stroma, hormones, metabolic products, pathologic processes (e.g., tumors), other immune cells and various soluble mediators (both host- and pathogen-derived) all contribute to this 3rd signal which can significantly influence the local dominance and activity of T cell subsets in a given local environment. While these factors are important in the development of local immune responses, their influence is layered upon the primary requirements for Signals 1 and 2. This broad spectrum of immunological mediators presents numerous targets for pharmacologic intervention, which may be aimed at broad intervention based on the primary signaling processes or fine-tuned to target specific tissues, processes or effector cell subpopulations.

In the next section, factors which contribute to the differentiation of these helper T cell subsets, along with transcription factors, effector molecules and effector functions will be discussed.

1.4.2 CD4⁺ Helper T Cell Populations

Helper T cell are $CD4^+$ lymphocytes whose primary function is to support and promote specific immune responses. An early step in adaptive immunity is naïve T cell recognition of antigens presented by antigen-presenting cells (APCs) in peripheral lymphoid organ. This interaction causes clonal expansion and differentiation of T cells into various $CD4^+$ T helper (T_H) subsets, including T_H1, T_H2 (Mosmann et al. 1986), T_H17 (Harrington et al. 2005), T_H9 (Chen et al. 2003; Dardalhon et al. 2008), follicular helper (T_{FH}) (Nurieva et al. 2009; O'Shea and Paul 2010) and regulatory T (Treg) cells (Chen et al. 2003). Early descriptions defined two populations; T_H1 cells which helped promote cell-mediated immune responses and T_H2 which helped promote humoral responses (Mosmann and Coffman 1989). Over the past two decades a wide array of additional $CD4^+$ helper T cell subsets have been investigated and defined, including T_H17, T_H9, T_H22, T_H25, regulatory T cells (including Treg, Tr1 and T_H3), and follicular helper T cells (T_{FH})) (Abbas and Lichtman 2015). For each of these subsets, there are defined factors which are responsible for inducing them, activation of specific STATs, lineage-specifying transcription factors, effector cytokines produced and defined functions (Caza and Landas 2015{Cools et al. 2007 #14,175). Additionally, different helper T cell subsets have distinct homing patterns, as well as specific expression of chemokine receptors and adhesion molecules. However, there is some plasticity in both the lineage commitment and cytokine secretion pattern of the helper cell subsets, which allows subsets to change with changing environmental conditions (O'Shea and Paul 2010; Oestreich and Weinmann 2012). Important helper T cell subsets are listed below (Abbas and Lichtman 2015; Tripathi and Lahesmaa 2014; Caza and Landas 2015; Zhu et al. 2010).

T_H1 cells contribute to cell-mediated immunity, macrophage activation and delayed-type hypersensitivity as well as clearance of intracellular pathogens and

enhancing the production of opsonizing antibody (particularly IgG) production by B cells. The cytokines produced for T_H1 effector functions are IFN-γ, TNF-α and lymphotoxin. Additionally, T_H1 cells express high levels of chemokine receptors CXCR3 and CCR5 and ligands for E-selectin and P-selectin. IL-12, IL-18 and type I interferons, commonly produced by macrophages, DCs and NK cells, are factors which induce this lineage, resulting in STAT4 and STAT1 signaling, and activation of T-bet as a lineage-specific transcription factor for T_H1 cells. Upon developing, T_H1 cells secrete IFN-γ which promotes further T_H1 differentiation and inhibits the differentiation of T_H2 and T_H17 subsets. T_H1 may also further stimulate IL-12 secretion by up-regulating CD40 ligand, which engages with CD40 on APCs to promote IL-12 production.

T_H2 cells contribute to humoral immunity, phagocyte-independent defenses, allergic responses, class switching of B cells (e.g., to IgE), and clearance of extracellular helminths and bacteria. IgE and eosinophil-mediated reactions can directly destroy helminths and cytokines produced by T_H2 cells increase mucus production and intestinal peristalsis, all of which help to eliminate intestinal helminths. The cytokines produced for T_H2 effector functions are IL-4, IL-13, IL-5 and IL-10. T_H2 cells express chemokine receptors CCR3, CCR4 and CCR8. IL-4 and indoleamine 2,3-dioxygenase induce this cell lineage, resulting in STAT6 activation. GATA-3 and c-MAF are lineage-specific transcription factors for T_H2 cells. T_H2 cells produce abundant IL-4, which is a signature cytokine of T_H2 cells that promotes additional T_H2 differentiation.

T_H17 cells are a relatively new addition to the helper T cell family that help promote the recruitment of neutrophils, are critical for destroying extracellular bacteria and fungi, provide protection at mucosal surfaces and play an important role in the clearance of *Mycobacterium tuberculosis* and *Klebsiella pneumoniae.* T_H17 cells, as their name suggests, produce IL-17, IL-17F, as well as, IL-6, IL-22, TNF-a and IL-10. Factors which induce this lineage include IL-6, IL-23, IL-1, MyD88 and low TGF-β levels. STAT3 is activated and lineage-specific transcription factors for T_H17 cells are RORγT and RORa. T_H17 differentiation is inhibited by IFN-γ and IL-4, therefore strong T_H1- or T_H2-promoting stimuli suppress T_H17 differentiation.

Treg cells (regulatory T cells) regulate and/or suppress existing immune responses and play important roles in maintaining tolerance and preventing or limiting autoimmune disease. There are numerous Treg populations, with $CD4^+CD25^+FoxP3^+$ being the most commonly studied and referenced, but additional $CD4^+$ Treg cells include Tr1 and T_H3, as well as other cell populations that are not within the $CD4^+$ subpopulation. Tregs largely produce the down-regulatory cytokines TGF-β and IL-10, depending on the subset and model system evaluated. They can reduce the ability of APCs to stimulate other T cells (e.g., by binding to CTLA-4) and regulate T cell populations by consumption of the T cell growth factor IL-2. High TGF-β, mTOR and various other factors can contribute to induction of this lineage. STAT5 signaling is activated and a lineage-specific transcription factor for most, if not all, $CD4^+$ Treg cells is FoxP3.

T_H9 cells provide protection at mucosal sites, particularly against helminth infections. Effector cytokines produced include IL-9 and IL-10. The cytokines IL-4 and

TGF-β induce these cells resulting in STAT6 activation. These cells have BATF as a lineage-specific transcription factor.

T_{FH} cells are present in lymphoid follicles, where they guide the migration of cells into germinal centers and help B cells produce high affinity and class-switched antibodies. The cytokine IL-21 and surface molecules OX40 and ICOS serve effector functions for these cells. Strong TCR signaling in conjunction with IL-12, IL-4, IL-21 and CXCR5 are factors which can contribute to this lineage, resulting in STAT3 activation. MAF (an IL-21 transactivator) is a lineage-specific factor for T_{FH} cells.

T_H22 cells contribute to host defense primarily at skin sites, where they protect against microbial pathogens and promote tissue repair or remodeling. Effector cytokines include Il-22, IL-13 and TNF-α, as well as, CCR10 and several fibroblast growth factors. IL-6, TNF-α and low TGF-β levels which promote the induction of T_H22 cells. STAT3 is thought to be activated and AHR is a key lineage-specific transcription factor.

T_H25 cells are a newly described population that plays an important role in mucosal immunity by limiting T_H1 and T_H17 induced inflammation, promoting IL-4 production by nonlymphoid cells and, in mice, promoting $CD4^+$ T cell memory. Several effector cytokines are produced by these cells, including IL-25, as well as other antibodies involved in various B cell functions and antibody production (i.e.,. IL-4, IL-5 and IL-13). IL-21, OX40 and ICOS are all effector cytokines of T_H25 cells, and IL-4 and IL-25 help induce these cells. Act1 is a lineage-specific transcription factor for T_H25 cells.

1.4.3 $CD8^+$ T Cell Activation and Function

$CD8^+$ cytotoxic T lymphocytes (CTLs or Tc1 cells) are the effector cells of the $CD8^+$ lineage. These cells play important roles in recognizing intracellular microbes and effectively eliminating infected cells or neoplastic cells expressing altered self-peptide in the context of class I MHC. $CD8^+$ T cells have TCRs which recognize antigen in the context of MHC class I. MHC class I is expressed on all nucleated cells and peptides expressed within the MHC class-I peptide binding groove are intracellular in origin. Thus, when pathogens are present intracellularly, some of their components become loaded onto MHC class I molecules and expressed on the cell surface. Naïve $CD8^+$ T cells recognize antigen in the context of MHC (Signal 1) and under the influence of appropriate co-stimulation (Signal 2) and microenvironmental influences (Signal 3) differentiate into effector cells within the lymph node or other induction sites. These effector cells then circulate and, when activated by antigen in peripheral tissues, can mediate CTL killing of target cells without further co-stimulation (Abbas and Lichtman 2015). Naïve $CD8^+$ T cells that encounter antigen and Signal 2 can proliferate without significant Signal 3 stimulation, but programming for clonal expansion (i.e., survival), effector functions and generation of memory populations relies on a 3rd signal which is usually IL-12 or type I IFNs (Mescher et al. 2006). The nature of this 3rd signal can influence the relative

functional of effector $CD8^+$ T cells, with type I IFNs playing a more important role in virally-related infections and IL-12 playing a more important role in pathogens which directly stimulate DC maturation (Mescher et al. 2006). Similar to $CD4^+$ T cell responses, optimal activation of naïve $CD8^+$ T cells requires prolonged exposure to Signals 1, 2 and 3 (Mescher et al. 2006).

Optimizing $CD8^+$ effector cell differentiation and function. The activation of $CD8^+$ T cells is optimal if antigen is presented by professional APCs such as DCs. Since DCs typically express low levels of MHC class I in contrast to other cells, optimal activation of $CD8^+$ T cells is accomplished by cross-presentation discussed earlier. Specifically, DCs ingest infected or tumor cells (or proteins expressed by these cells) and transfer these proteins from what would normally be the MHC class II pathways to enter the MHC class I pathway. $CD8^+$ and $CD103^+$ DCs are the DC populations most efficient at cross-presentation. $CD4^+$ helper T cells are also important in helping naïve $CD8^+$ T cells achieve full activation and differentiation into functional CTLs and memory cells. IL-2, IL-12 and type I IFNs all promote the differentiation of naïve $CD8^+$ T cells into CTLs, while IL-15 and IL-21 promote the induction, maintenance and survival of CTLs and memory $CD8^+$ T cells (Abbas and Lichtman 2015; Mittrucker et al. 2014). Development of memory T cells (both $CD4^+$ and $CD8^+$) is supported by IL-7 and IL-15, which induce anti-apoptotic proteins and stimulate low-level proliferation. The differentiation of naïve $CD8^+$ T cells is also influenced by a transcriptional network where T-bet, Id2, Blimp-1 and (IRF)4 promote effector development, and Bcl-6, eomesodermin (Eomes), Id3, TCF-7 and Foxo1 promote the differentiation of memory cells (Mittrucker et al. 2014). As with $CD4^+$ T cell subpopulations, there are numerous $CD8^+$ T cell subpopulations. T_C1, T_C2, T_C17, T_C9 and $CD8^+$ Tregs have all been described and reviewed (Mittrucker et al. 2014). Lineage plasticity among the various $CD8^+$ T cell populations is recognized as described for $CD4^+$ T cells, with T_C1 and $CD8^+$ Tregs being the most stable of the populations (Caza and Landas 2015; Coomes et al. 2013; O'Shea 2013).

CTL effector (killing) functions: $CD8^+$ T cells mediate killing through two main mechanisms following antigen recognition of target cells. The first process is mediated by the exocytosis of granules containing perforin and granzyme. These granule contents are released into the immune synapse and taken up by the target cell endosome and released into the cytosol, where they activate the caspases that result in apoptotic cell death. NK cells utilize essentially the same mechanism of cytotoxicity to mediate killing (Abbas and Lichtman 2015). The second process is Fas/Fas-ligand-mediated cell killing where FasL on the CTL interacts with death receptor Fas on the target cell, and the Fas/FasL ligation activates the chain of processes that result in apoptosis. $CD8^+$ T cells may express additional receptors which enhance or regulate killing activity, including receptors of the killer immunoglobulin receptor (KIR) family and NKG2D receptor. KIR receptors recognize ligands and MHC I molecules present on cells that are infected with microbes, are transformed (e.g., neoplastic), or have undergone stress-associated alterations. Most KIRs are inhibitory, although some activate cytotoxicity activity. NKG2d is an activated receptor which binds class I MHC-like proteins found on virally-infected cells and tumor cells but not normal cells (Abbas and Lichtman 2015).

Inducing cell-mediated immune responses at local sites and potential exhaustion. $CD8^+$ T cells further enhance immune activation and cell-mediated immune responses by producing IFN-γ which activates and enhances the phagocytic function of macrophages. Since $CD8^+$ T cell effector functions are mediated locally, it is particularly important that they migrate to the site of infection. $CD8^+$ T cells leave the lymph nodes by down-regulating CD62L and CCR7,and it is hypothesized that tissue-specific combinations of selectins, cytokines and integrins act as homing markers or local "area codes" to facilitate migration to appropriate sites (Gerritsen and Pandit 2016). It is at these local sites that the $CD8^+$ T cells can mediate their killing actions. However, in the presence of persistent antigen exposure, chronic viral infection or expression of immunoregulatory molecules, $CD8^+$ T cells can undergo exhaustion or become tolerant, similar to the process seen in $CD4^+$ T cells. One example of this is the chronic antigen exposure to tumor antigens which, in addition to the presence of the immunoregulatory PD-1 molecule, contributes to the exhaustion of $CD8^+$ T cells (Abbas and Lichtman 2015). Many cancer immunotherapies aim to revive the exhausted or reverse the tolerant immune responses, as will be discussed later in this chapter.

1.4.4 B Cells: Activation, Antibody Production and Generation of Humoral Responses

Capture of external antigens by B cells, incorporation of antigenic peptides into MHC class II molecules and subsequent presentation to $CD4^+$ T_H cells is a crucial process in adaptive immunity (Mitchison 2004). The T cell/B cell interaction allows formation of germinal centers in lymphoid follicles, and leads to the formation of high-affinity antibody-producing plasma cells and antigen-specific memory B cells. B cells participate in this process by capturing external antigens via their B cell receptor (BCR) (surface Ig), processing of antigens through the endosomal/lysosomal compartment, loading of antigenic peptides onto MHC class II molecules, and presentation of the MHC/antigenic peptide complexes to $CD4^+$ T_H cells.

B cells originate in the bone marrow, then migrate to the spleen to undergo development into mature naïve B cells. The naïve B cells then take up residence in secondary lymphoid organs such as spleen or lymph nodes, which provide the environment necessary for the B cells to become fully activated (von Andrian and Mempel 2003; Fig. 1.6). Naïve B cells enter lymph nodes via high-endothelial venules and migrate to the primary lymphoid follicles in the cortical region of the lymph node. Migration of the naïve B cells within the lymph node is controlled by the interaction of CXC-chemokine ligand 13 (CXCL-13) on follicular stromal cells and CXC-chemokine receptor 5 (CXCR5) on naïve B cells (Yuseff et al. 2013). Naïve B cells encounter particulate antigen that is presented by macrophages, follicular dendritic cells, or dendritic cells (Carrasco and Batista 2007; Junt et al. 2007; Suzuki et al. 2009; Qi et al. 2006). Soluble antigenic peptides may enter the lymph

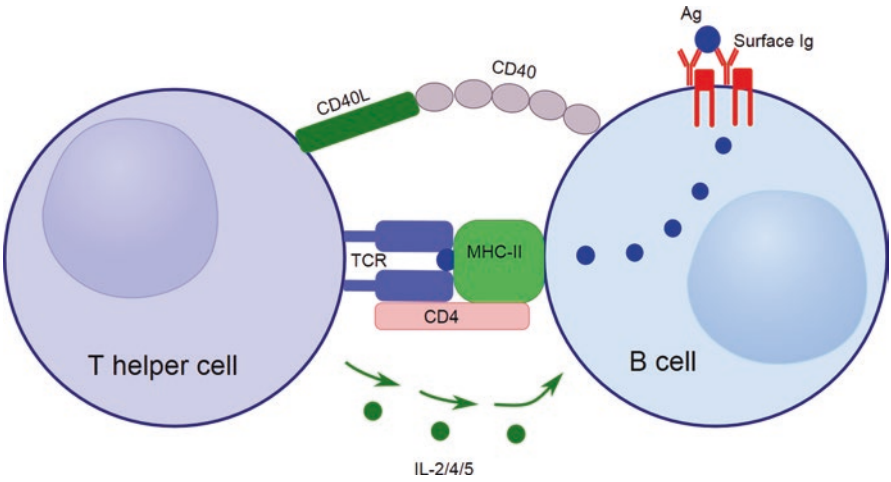

Fig. 1.6 B cell–T cell conjugate. Antigen recognition by surface immunoglobulin on B cells provides a pathway by which antigen is processed and presented in MHC context to helper T cells. Downstream events lead to expansion of antigen-specific lymphocyte populations as well as class switching and affinity maturation of B cells, thus providing multiple targets for pharmacological intervention. See text Sect. 1.4.4 and associated references for additional details

node independently of cell-mediated antigen presentation (Pape et al. 2007), either by simple diffusion into the lymph node (Gretz et al. 2000) or penetration of small pores in the subcapsular sinus of lymph nodes (Clark 1962).

Activated B cells within germinal centers have highly polarized morphology, with filopodia at the leading edge and uropods at the trailing edge of migrating B cells (Yuseff et al. 2013). Stationary B cells extend protrusions that interface with FDCs to sample their antigen load (Hauser et al. 2007). This polarization of B cells within secondary lymphoid organs is crucial to the function of B cells and the eventual humoral immune response, thus is a potential target for pharmacologic manipulation.

Antibodies (also called immunoglobulins): Antibodies are produced by B cells and exist either as membrane-bound antibodies on the surface of B cells where they function as antigen receptors or as secreted antibodies which neutralize toxins and help eliminate microbes. Elimination of microbes can occur via activation of the complement system, enhancing phagocytosis via opsonization of pathogens, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-mediated mast cell activation. Antibodies share similar basic structure but, like T cell receptors, are highly variable in the antigen-binding regions (Abbas and Lichtman 2015). Antibodies have a symmetric core comprised of two identical light chains and two heavy chains where each of these chains have Ig domains. Ig domains structurally are two layers of a beta-pleated sheet held together by a disulfide bridge with strands of the beta-sheet connected by short loops. Antibodies have a "Y" shape with an Fc region base and two Fab region "arms" containing multiple Ig domains. The most distal amino-terminal variable (V) region of the Fab portion is what interacts with

antigen and where the antigen specificity of any given cell is determined. The antigen recognition areas of the heavy and light chains each have three hypervariable regions, also known as complementarity determining regions (CDRs). Amino acid differences in the hypervariable CDRs are responsible for the diversity in antigen recognition. The Fc region has binding sites for several important effector molecules, including complement C1q and Fc receptors.

Rearrangement of antigen receptor genes occurs during lymphocyte development and involves site-specific rearrangement of the V region with the diversity (D) and joining (J) regions in a process called V(D)J rearrangement. A similar process for generating diversity exists for B cell receptors (BCR) and T cell receptors (TCR). The remaining Ig domains distant from the antigen-binding site within the antibody comprise the carboxy-terminal constant (C) regions and it is the heavy chain C region which dictates the distinct class (isotype) and subclass of the antibody produced by an individual cell.

The five classes of mammalian immunoglobulin molecules have structural and functional attributes that are of particular interest to toxicologists and toxicologic pathologists. The antibody isotypes include IgA, IgD, IgE, IgG and IgM, each of which has unique properties and functions (Abbas and Lichtman 2015). Monomeric immunoglobulin G (IgG), which is the primary immunoglobulin in serum and non-mucosal tissues, directly inactivates pathogens and initiates additional downstream signaling via the Fc and complement receptors on the Fc region. IgG has numerous roles in immune responses, including ADCC, opsonization and complement activation, which are critical for removing pathogens. Pentameric immunoglobulin M (IgM) in the serum is particularly efficient at complement activation, and monomeric, membrane-bound IgM is the major immunoglobulin involved in B cell recognition of antigens. Immature B cells express IgM (and often co-express IgD). B cells can switch the isotype from IgM to other isotypes, such as IgG, IgE or IgA through a process called immunoglobulin class switching (or class-switch recombination) which is primarily under the influence of cytokines. Immunoglobulin D (IgD) exists primarily in a membrane-bound form on the surface of B cells, where it serves along with IgM in activation of B cells. IgE plays important roles in defense against helminth parasites, as well as immediate hypersensitivities and allergies. Serum immunoglobulin A (IgA), which exists in monomeric and dimeric forms, links pathogens to effector cells via Fc receptors specific for IgA. Secretory dimeric IgA is important in protection of mucosal surfaces against pathogen invasion. Both serum and secretory dimeric IgA molecules are connected by a protein known as J chain, and the secretory form of dimeric IgA has an additional protein known as secretory component that protects the IgA molecule as it transitions across the epithelium of mucosal surfaces. It has been estimated that the daily production of IgA is the highest of any of the immunoglobulin subtypes, which is a reflection of the critical role that IgA plays in protection against enteric pathogens.

In common parlance it is said that antibodies bind to antigens. To be precise, the CDR on the immunoglobulin molecule functions as a *paratope* that binds to an *epitope* on the antigen. The *agretope* is the peptide sequence on the antigen that binds to the *desetope* in the antigen-binding cleft on the MHC molecule. The immunologic

response typically is directed at a relatively short sequence of the total polypeptide chain, therefore antibodies of multiple specificities may be directed toward a single protein. Thus different antibody clones that are raised to a single polypeptide may exhibit quite different immunoreactivity in a laboratory setting, e.g., immunohistochemical staining. In addition, antibodies raised to a single epitope may react with a completely different polypeptide contained in a different tissue. This latter feature is the basis for the tissue cross-reactivity assay. Immunological binding is based on the three-dimensional structure of the epitope, not the chemical sequence, which introduces the possibility of molecular mimicry between molecular sequences that have similar three-dimensional profiles. Monoclonal antibodies are those that have identical CDRs and recognize the same epitope of any given antigen while polyclonal antibodies recognize different epitopes, even if against the same pathogen and the polyclonal nature is due to differences in the CDRs of the V region. Within drug development, there are numerous applications and interest in therapies utilizing monoclonal antibodies or various antibody fragments (Nelson and Reichert 2009; Liu 2014). There are approximately 22 monoclonal antibodies currently in clinical use in cancer, inflammatory or immune-mediated diseases (Abbas and Lichtman 2015). There is an abundance of literature to provide detailed information regarding immunoglobulin structure and function that can be useful not only in understanding basic biology but also as a foundation for developing antibody-based therapies (Schroeder et al. 2013; Owen et al. 2013; Delves et al. 2006).

1.5 Regulation of the Immune Response

1.5.1 Cytokines

Cytokines are secreted proteins that regulate and coordinate not only the differentiation of various T cell subsets but also refine and control numerous aspects of immune responses. It is complex interplays rather than the simple concentration of cytokines that influence processes such as helper T cell differentiation (Huang and August 2015; Schmitt and Ueno 2015). Cytokines have numerous names and can be designated as interleukins, lymphokines, chemokines, interferons and colony-stimulating factors, with there being some overlap with actions of hormones or growth factors). Cytokines have numerous functions and can serve as pro-inflammatory or anti-inflammatory factors, growth factors or transcription factors and provide a wide range of effects on various cells and tissues (immune or otherwise). Chemokines are chemoattractant cytokines important in regulating immune cell trafficking and recruitment (Abbas and Lichtman 2015). Cytokines can largely be grouped into four types; The IL-1 family (e.g., IL-1 and IL-18), the IL-17 family (IL-17A, IL-17B, etc.), the transforming growth factor beta superfamily (e.g., TGF-β1, TGF-β2 and TGF-β3) and the largest four-α-helix family which contains the IL-2, interferon (IFN) and IL-10 subfamilies. Cytokines mediate their actions through interactions with cytokine receptors, which are grouped according to their

three-dimensional structure. Upon binding to a receptor, cytokines initiate intracellular signaling events which impact genes, commonly via their transcription factors, and subsequently influence cellular functions.

It is through the finely orchestrated interactions between cytokines and immune cells that normal immune responses occur. Dysregulation of cytokine levels have been linked to inflammatory and autoimmune disease, as well as Alzheimer's disease, cancer and numerous other disease states (Hirahara and Nakayama 2016; Dranoff 2004; Landskron et al. 2014; Swardfager et al. 2010). The fact that the many of the drugs used for treatment of inflammatory or autoimmune diseases target cytokines or their receptor demonstrates the importance of cytokines in pathological conditions. Effects of therapeutics and immunotherapeutics on cytokines are an important consideration in drug discovery and development, and there is an increasing focus on assessing the safety and immunotoxicity of immunomodulatory therapies (Brennan et al. 2010; Kawabata and Evans 2012; Ramani et al. 2015). Inadvertent triggering and subsequent dysregulation of this system can cause significant pathology. Increasingly, cytokines are being used as biomarkers of toxicity along with other immunotoxicology assays (Kawabata and Evans 2012; Ramani et al. 2015).

The major cytokines groups, which are largely based on based on functionality, consist of interferons, interleukins, chemokines, tumor necrosis factor and colony-stimulating factors. Interferons play important roles in activation of antiviral properties and regulation of innate immune responses. Interleukins guide growth and differentiation of leukocytes while chemokines (chemoattractant cytokines) control leukocyte recruitment. Tumor-necrosis factor (TNF-α) activates cytolytic T cells and is primarily considered pro-inflammatory, while colony-stimulating factors stimulate hematopoietic precursors to proliferate and differentiate. Cytokines are often grouped according to relative pro-or anti-inflammatory (i.e., regulatory) functions, although this over-simplification does not represent the complexity or nuances of cytokines in various pathological and physiological states. Thus, when evaluating and interpreting cytokine levels, and whether a particular cytokine may be contributing or minimizing disease, it is important to understand both the basic biology and normal function of the cytokine within the model system evaluated, as well as potential therapeutic influences may impact the levels of that cytokine along with other complementary, antagonistic, redundant or associated cytokines. During drug development a full understanding of disease pathogenesis and downstream effects of a potential therapy may be difficult to comprehend in the context of a single nonclinical safety study.

The impact of cytokines on helper T cell differentiation has been discussed and a detailed description of all of the various cytokines involved in a dizzying array of immune responses in various organs and various disease states is beyond the scope of this chapter. Although their role in disease states are often a focus of many studies and reviews, cytokines are important regulators involved in maintaining homeostasis (Khaled and Durum 2002). For example, cytokines such as TGF-β, IL-10 and IL-2 play crucial roles in maintaining gut homeostasis and tolerance (Abbas and Lichtman 2015). Disrupted levels of these cytokines or an altered balance tipped

towards inflammatory cytokines can influence the health of an individual. Often the set of pro-inflammatory cytokines are dictated by the disease pathogenesis of interest. Specifically, allergic diseases are considered to be primarily T_H2-mediated with elevations of IL-4, IL-13 and other Th-2 related cytokines contributing to disease exacerbations. In contrast, in inflammatory or autoimmune diseases that have a T_H1-mediated pathogenesis (e.g., multiple sclerosis) , cytokines such as IL-12, IL-23, IFN-γ and IL-17 can all contribute to disease exacerbations (Hirahara and Nakayama 2016). Many diseases have a combination of T_H1, T_H2, T_H17 and/or other responses contributing to disease pathogenesis, which results in challenges in understanding disease pathogenesis and treating the diseases (Hirahara and Nakayama 2016). Some pathologic processes involve a cytokine storm that may include TNF-α, type I interferons (IFN-α/β), IFN-γ, IL-1α/β, IL-6 and CCL2 (Tisoncik et al. 2012). Following is a brief presentation of the more commonly evaluated cytokines involved in various physiological and pathophysiological states.

IFN-γ is a type II interferon, typically considered a pro-inflammatory cytokine, which is produced in large part by NK cells and then later by T_H1 cells to contribute to cell-mediated responses. IFN-γ activates macrophages to kill phagocytosed microbes, promotes switching to specific IgG subclasses (e.g., IgG2a or IgG2c in mice), inhibits IL-4-dependent isotypes (i.e., IgE) and promotes the expression of proteins that increase MHC-associated antigen presentation and amplification of T cell-dependent immune responses. As part of its role in cell-mediated immune responses is can help stimulate the differentiation of naïve $CD8^+$ T cells into effector CTLs (Abbas and Lichtman 2015).

IL-1 family: The IL-1 family of cytokines includes IL-1α, IL-1β, IL-1 receptor antagonist, IL-18 and IL-33. IL-1 is produced by several cell types including macrophages, DCs, fibroblasts, endothelial cells and others. IL-1 activates endothelial cells, promotes inflammation and coagulation and, along with IL-6, promotes fever. **IL-18** is a member of the IL-1 family of cytokines that is produced by numerous cells (immune and otherwise). It promotes IFN-γ synthesis in NK and T cells and pro-inflammatory cytokines such as TNF-α, IL-1β and GM-CSF in monocytes and activates neutrophils**. IL-33** induces helper T cells, mast cells, eosinophils and basophils to produce T_H2 cytokines.

IL-2, a member of the four-alpha-helix bundle family, is an essential development cytokine that is critically important role in promoting the proliferation of both $CD4^+$ (helper) and $CD8^+$ T cells and supports their differentiation into effector and memory T cells. In particular, IL-2 plays an important role in Treg differentiation (Abbas and Lichtman 2015).

IL-4 is a member of the four-alpha-helix bundle family that plays an important role in T_H2 responses. T_H2 cells produce IL-4 to promote additional T_H2 cells in a positive feedback loop, as well as, down-regulating the T_H1-inducing cytokines IFN-g and IL-12. IL-4 is a key regulator in promoting humoral immune responses and it promotes B cell Ig heavy chain class switching to IgE isotype. It also promotes switching to IgG4 (in humans) or homologous IgG1 (in mice), inhibits IgG2a and IgG2c, and up-regulates MHC class II production. IL-4, in conjunction with related IL-13, can promote the alternative form of macrophage activation and stimulate

peristalsis in the gut, and both IL-4 and IL-13 stimulate recruitment of leukocytes (especially eosinophils) (Abbas and Lichtman 2015).

IL-5 is an activator of eosinophils and is a principal link between T cell activation and eosinophilic inflammation. Its primary actions are to activate mature eosinophils and stimulate the growth/differentiation of eosinophils and to stimulate B cell growth and immunoglobulin secretion (Abbas and Lichtman 2015).

IL-6 is secreted by T_H2 cells as well as macrophages, and during T_H2 cell differentiation plays a relative anti-inflammatory role. However, IL-6 is also a mediator of fever and the acute phase response, thus has a role in numerous inflammatory and autoimmune diseases.

IL-9 plays an important role in stimulating cell proliferation and preventing apoptosis. Although studies of IL-9 are limited compared to other cytokines, it's thought to play a role in T_H2-mediated diseases (Kaplan et al. 2015; Goswami and Kaplan 2011).

IL-12 is primarily produced by phagocytic cells including macrophages, DCs and neutrophils. It is considered an inflammatory cytokine that is important in promoting differentiation of T_H1 cells, promoting IFN-γ and TNF-α synthesis from NK cells, reducing the effects of IL-4 on IFN-γ production and increasing cytotoxic activities in NK cells and T (i.e., $CD8^+$) cells (Abbas and Lichtman 2015).

IL-13 is functionally & structurally similar to IL-4, and interfaces with a receptor that is a heterodimer of the IL-4Rα and IL-13Rα 1 chain. Similar to IL-4, IL-13 is secreted by T_H2 cells, induces IgE production by B cells, and is thought to be an even more central mediator in allergic inflammation than IL-4. IL-13 increases mucus secretion by airway and gut epithelial cells (Abbas and Lichtman 2015).

IL-15 is a member of the four-α-helix bundle family and is secreted by a wide array of cell types and tissues. It induces proliferation of NK cells and is important for survival of memory T cells by preventing apoptosis (Abbas and Lichtman 2015).

IL-17 is a pro-inflammatory cytokine that is induced by IL-23 and produced by T_H17 cells. First described in 2006, it has emerged as an important player in the development of inflammatory and autoimmune disease. It recruits monocytes and neutrophils to inflammatory sites similar to IFN-γ. IL-17 induces neutrophil-rich inflammation (through stimulating TNF production and chemokines) and increases G-CSF and G-CSF receptor expression to enhance neutrophil generation. It also plays an important role in delayed-type reactions and may act synergistically with other inflammatory cytokines. It also stimulates the production of antimicrobial substances, including defensins (Abbas and Lichtman 2015; Onishi and Gaffen 2010).

IL-21 is a member of the four-alpha-helix bundle family that is produced by activated $CD4^+$ T cells. It is required for the generate of T_{FH} cells and the stimulation of B cell germinal cells. It increases the proliferation, differentiation and effector functions of NK cells and $CD8^+$ T cells and plays a role in induction of $CD8^+$ T cell memory and prevention of $CD8^+$ T cell exhaustion (Abbas and Lichtman 2015).

IL-22 Is an α-helical cytokine that binds heterodimeric cell surface receptors composed of IL-10R2 and IL-22R1 subunits. It is produced by activated T cells, especially T_H17 cells, but also epithelial cells, where it plays a major role in

maintaining epithelial integrity (promoting barrier function and stimulated repair) and stimulating the production of chemokines and anti-microbial peptides by epithelial cells (Abbas and Lichtman 2015).

IL-10 is considered an anti-inflammatory cytokines with numerous regulatory and anti-inflammatory characteristics, although it can also have a positive role in inflammation. It is produced primarily by monocytes and less so by Treg, T_H2 and other immune cells. It has an important role in maintaining tolerance and numerous sites (e.g., the gut) It inhibits activated immune cells (particularly DCs and macrophages). IL-10 has many functions which include shifting Th0 differentiation away from the T_H1 pathway, down-regulation of MHC class II and co-stimulatory moleculeexpression on APCs, and promotion of B cell function and antibody production. Its role in inflammation is complex and is primarily influenced by relative ratios of IL-10 to IL-12 and TNF-α, as well as its ability to promote T_H2-mediated disease states (Abbas and Lichtman 2015; Couper et al. 2008).

IFN-α, and IFN-β are type I interferons that play an important role in early innate immune responses. They are increased following viral infection and help promote the innate immune response, increase MHC class I expression, NK cell activation and T_H1 induction.

TNF -alpha is considered a pro-inflammatory cytokine and an important cytokine of the acute phase reaction. It is produced primarily by macrophages but NK cells, T cells and numerous other cell types can produce it. The primary function of TNF-α is to regulate immune cells. It activates neutrophils, promotes inflammation, promotes activation of endothelial cells (i.e., inflammation and coagulation) and is an endogenous pyrogen, inducing fever and apoptotic cell death, inflammation and cachexia. The TNF superfamily of cytokines includes not only TNF but also lymphotoxin-α, lymphotoxin-αβ, BAFF, APRIL and osteoprotegrin) (Abbas and Lichtman 2015).

TGF-β is secreted by numerous cell types and has a complex production and secretion pathways. TGF-β is widely considered an anti-inflammatory or regulatory cytokine due to its ability to inhibit the effector function and proliferation of T cells, inhibit classical activation of macrophages, and activate neutrophils and endothelial cells. It also regulates the differentiation of various T cell subsets, most specifically the balance between T_H17 and Treg cells. TGF-β inhibits B cell proliferation but also stimulates class switching to IgA, which contributes to its protective effects within the gut. TGF-β also plays an important role in wound healing by stimulating fibroblasts to produce collagen and stimulating angiogenic factors (Abbas and Lichtman 2015).

1.5.2 Chemokines

The first chemokines, CXCL8 (IL-8) and CCL21 (MCP-1) were discovered in the late 1980s, and several additional chemokines were identified in the early 1990s. This first wave of chemokines had a largely pro-inflammatory role in innate

immunity. Chemokine: chemokine receptor interactions in this first group of chemokines were found to be promiscuous, with some chemokines binding to multiple receptors and some chemokine receptors accepting multiple chemokines (Zlotnik and Yoshie 2012). Further investigations revealed an additional group of chemokines that are involved with lymphocyte and dendritic cell homeostasis, thus have a role in adaptive immunity. This second group of homeostatic chemokines have less promiscuous interactions with their receptors, and serve as 'master regulators' of the lymphocytes and dendritic cells that function in adaptive immunity (Zlotnik et al. 2006; Moser et al. 2004).

The key molecular feature of chemokines is the presence of four conserved cysteine residues that form two disulfide bonds, one pairing the first and third cysteines and the second pairing the second and fourth cysteines. Based on the arrangement of the N-terminal double cysteine residues, chemokines are grouped into four subfamilies: CXC, CC, (X)C, and CX3C. X represents a variable amino acid residue, thus CXC chemokines have one amino acid between the two terminal cysteines, CX3C chemokines have three amino acid residues between the terminal cysteines, CC have no amino acids between the adjacent cysteines, and the first and third cysteines are missing in the (X)C subfamily.

Chemokines may also be grouped according to function (Zlotnik and Yoshie 2012; Moser et al. 2004). Inflammatory chemokines are in group 'I', homeostatic chemokines are in group 'H', dual-purpose chemokines are in group 'D', and chemokines present in plasma are in group 'P' (Moser et al. 2004; Nomiyama et al. 2011). Another group of chemokines stored in the α-granules of platelets are quickly released upon platelet activation (Flad and Brandt 2010).

Chemokine receptors are grouped into four subfamilies, based on the subfamily of their major chemokine ligands (Zlotnik et al. 2006). To date 18 chemokine receptors with standard chemotactic activity have been identified in humans and mice (Zlotnik and Yoshie 2012). In general, chemokine receptors of inflammatory ('I') chemokines exhibit a greater level of promiscuity in binding than do chemokine receptors of the homeostatic ('H') or dual-purpose ('D') groups (Nomiyama et al. 2011).

Five additional atypical, nonchemotactic chemokine receptors serve as chemokine scavengers, decoy receptors or transporters (Mantovani et al. 2006; Ulvmar et al. 2011). DARC (Duffy antigen chemokine receptor), a classic example of an atypical chemokine receptor, binds multiple inflammatory chemokines but not homeostatic chemokines. DARC is abundantly expressed on erythrocytes, and serves as a chemokine sink. DARC is also expressed on endothelial cells, where it mediates chemokine transcytosis from the basolateral aspect to the luminal surface of endothelial cells, thus promoting emigration of leukocytes at sites of inflammation (Pruenster and Rot 2006).

Inflammatory chemokines are involved in the control of inflammatory reactions, and homeostatic chemokines are important in the control of immune responses, thus modulation of chemokine/chemokine receptor interactions are attractive drug development targets. Development of chemokine-related drugs is hindered to some extent by the substantial lack of homology between human and rodent chemokines, there-

fore suitable animals models are not readily available for experimentation. Despite these obstacles, chemokine-related pharmaceuticals are being developed for inflammatory bowel diseases (CCR9 antagonists) (Proudfoot et al. 2010), various T_H1 cell responses (CXCR3 antagonists) (Liu et al. 2011), and T cell leukemia-lymphoma (CCR4) (Tobinai et al. 2012).

Fractalkine, the sole member of the CX3C family, consists of a membrane-bound chemokine domain attached to the cell membrane by a mucin-like stalk (Bazan et al. 1997). Under homeostatic conditions, fractalkine is expressed primarily by neurons and epithelial cells of the lung, kidney and intestine (Kim et al. 2011; Lucas et al. 2001). During inflammation it is also expressed by endothelial cells and vascular smooth muscle cells (Garcia et al. 2000; Ludwig et al. 2002). Fractalkine is the unique ligand for the chemokine receptor CX3CR1, which is expressed on monocytes, NK cells, T cells and smooth muscle cells (Imai et al. 1997; Lucas et al. 2003). There is evidence that CX3CR1 conveys an essential survival signal to monocytes and macrophages, and has anti-apoptotic and proliferative effects on vascular smooth muscle cells, thus contributes to the progression of atherosclerosis (White et al. 2010; Landsman et al. 2009; Lesnik et al. 2003; Combadiere et al. 2003). Interception of these fractalkine-mediated effects is a potential pharmaceutical target for treatment of atherosclerosis, which is a major cause of human fatalities. See (Zlotnik and Yoshie 2012) for a review of chemokine immunobiology, and (White and Greaves 2012) for a review of fractalkine.

1.5.3 Interferon Type I

There are three distinct interferon (IFN) families. The type I interferon family consists of multiple (13 in humans, 14 in mice) cytokines of the IFNα subtype, a single IFNβ, and several poorly defined single gene products (Pestka et al. 2004). The type II interferon family consists only of IFNγ, which is predominantly expressed by T cells and NK cells (Schoenborn and Wilson 2007). The type III interferon family consists of IFNλ1, IFNλ2, IFNλ3, and IFNλ4 (Prokunina-Olsson et al. 2013; O'Brien et al. 2014), which have functions similar to the type I interferons except the IFNλ receptor is largely restricted to epithelial cell surfaces (Witte et al. 2010).

The best-known members of the type I interferons are IFNα and IFNβ, which have antiviral activity in virus-infected cells as well as cells located near the virus-infected cells (Yan and Chen 2012). In addition to antiviral effects, the type I interferons are involved in multiple innate and adaptive immune responses, which include promoting $CD4^+$ and $CD8^+$ T cell responses and enhancing the responses of dendritic cells and monocytes, NK cell responses, and B cells (McNab et al. 2015). In addition to positive effects on immunity, type I inferons can have negative effects due to immunosuppression (Biron 2001) or provoking inflammation and tissue damage (Davidson et al. 2014).

Most cells can produce IFNα/β, typically in response to stimulation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and retinoic acid-

inducible gene 1 (RIG-1) (McNab et al. 2015). TLR4 on cell surfaces, which recognizes bacterial lipopolysaccharide, is the most potent inducer of type I IFN. TLR3, TLR7/8 and TLR9 in endosomal compartments respond to double-stranded RNA, single-stranded RNA and unmethylated CpG DNA, respectively (Moynagh 2005). Binding of type I IFN to its receptor initiates signaling through a variety of signaling pathways, including the STAT1-STAT2-regulatory factor 9 (IRF9), STAT1 homodimers, mitogen-activated protein kinases (MAPKs), and the phosphoinositide 3-kinase (PI3K) pathway, resulting in a variety of effects on cell function (McNab et al. 2015).

See (McNab et al. 2015) for a review of type I interferon activities in infectious disease.

1.5.4 Extracellular Vesicles/Exosomes and Immunity

There is a large body of evidence that cells of many types release membrane-bound extracellular vesicles that are involved in various forms of intercellular communication, including communication between cells of the immune system. The microvesicles and nanovesicles released by most types of cells vary in size and the mechanism of generation (Thery et al. 2002; Thery et al. 2009; Yang and Robbins 2012; EL Andaloussi et al. 2013). Microvesicles are typically larger than 0.2 μm in diameter and are formed by budding or shedding from the plasma membrane. Nanovesicles (exosomes) are 30–100 nm in diameter and are formed by inward budding of the peripheral membrane of multivesicular bodies or late endosomes, which results in intraluminal vesicles inside the endosomes. At this point the multivesicular bodies laden with intraluminal vesicles (ILV) may (1) fuse with lysosomes and release their cargo into the lysosomal lumen for degradation or (2) fuse with the plasma membrane of the cell and release their cargo as exosomes in the extracellular fluid. The exosomes contain various proteins, mRNA, miRNA and extra-chromosomal DNA fragments that can function in intercellular communication.

Extracellular vesicles derived from antigen-presenting cells (APCs) retain the surface MHC molecules of the parent cell, and present various antigenic peptides for recognition by cognate T cells. Extracellular vesicles released by B cells have surface expression of MCH class II plus the co-stimulatory and adhesion molecules that allow them to directly stimulate $CD4^+$ T_H cells (Raposo et al. 1996). In addition to direct stimulation of T cells, extracellular vesicles can indirectly stimulate T cells through transfer of their antigenic peptides to other APCs such as dendritic cells. The transferred MHC-antigenic peptide complexes may be degraded by the recipient APC and the peptides expressed in the context of the MHC molecules of the recipient APC. In addition, MHC-peptide complexes on the surface of extracellular vesicles may be transferred directly to the surface of the recipient APC, without internal processing. This latter process is known as ‘cross-dressing’ (Robbins and Morelli 2014).

1.6 Refinement of Immune Responses

Detailed communication between cells and soluble mediators of the immune systerm result in the induction of immune responses. Further interactions can help refine an immune response and contribute to the development of an amnestic (memory) response. Affinity maturation and the development of immunological memory are two such processes.

1.6.1 Affinity Maturation

The initial antigen recognition and response by B cells bearing cognate receptors is followed by an affinity maturation process that is nearly unique in all of biology. Affinity maturation takes place largely in germinal centers of lymph nodes and the spleen, which have been called 'Darwinian microcosms' due to the extraordinary selective processes that take place in these microscopic structures (Kelsoe 1998). An enzyme complex that targets variable regions of Ig genes causes successive cycles of mutation in the variable region genes of B cell receptors (BCR) of proliferating B cells within germinal centers, resulting in an extremely high rate of mutagenesis in the subpopulation of B cells that expressed specific receptor for the antigen and interacted with T_H cells with similar antigen specificity. The mutated genes are translated to produce altered proteins that are incorporated into the surface immunoglobulin receptors on B cells. Selective processes within the germinal center allow for survival and proliferation of those B cells that possess receptors of the highest affinity, while those B cells with the lower affinity receptors die by apoptosis. Repetitive cycles of this process within germinal centers result in a population of B cells that express high affinity for the cognate antigen that initiated the process. This population of high-affinity antigen-specific B cells is expanded in germinal centers of B cell follicles in the lymph nodes and spleen. As a result, the average affinity of BCRs and secreted antibodies produced near the end of the humoral immune response is higher than the average affinity at the beginning of the immune response. For details of germinal center involvement in immunobiology, see (De Silva and Klein 2015; Victora and Nussenzweig 2012).

1.6.2 Generation of Immunological Memory

Specificity and immunological memory are two key characteristics of the adaptive immune system. The development and implementation of immunological specific is covered in other sections. This section provides a brief overview of the development and homeostasis of immunological memory.

The evolution of the immune system has included a continual increase in complexity which provides the host with a survival advantage over infectious agents. The development of immunological memory allows a system for rapid and substantial response to repeated pathogen exposures, thus resulting in long-lasting protection to commonly encountered infectious organisms. The generation of subsets of memory T cells with distinct homing and functional properties increases the specificity of immunological defenses (Lefrancois and Marzo 2006).

1.6.2.1 Memory B Cells

The antibodies that neutralize and clear pathogens are a critical component of protection against infectious diseases, and the existence of immunological memory directed toward common pathogens affords the host with an important survival advantage. The expansion of antigen-specific B cell populations in secondary lymphoid organs allows an immediate humoral response to invasion by pathogens, but B cells can also develop into memory B cells and memory plasma cells that provide protection over an extended period of time (Yoshida et al. 2010). Memory B cells do not have a constitutive effector function, and require restimulation (i.e., a second pathogen exposure) before they can contribute to the memory response. By contrast, memory plasma cells exist in the bone marrow as long-lived, nonreplicative cells that continually produce large quantities of specific antibodies (Manz et al. 1997; Slifka et al. 1998). They do not require restimulation to release the immunoglobulins, and have lost their ability to recognize specific antigen (Manz et al. 1998). The antibodies produced by memory plasma cells may result in very long-lived immunity. Immunity following smallpox vaccination is known to persist more than 50 years (Crotty et al. 2003; Hammarlund et al. 2003), and specific antibodies produced by memory plasma cells have been shown to have half-lives of 50–200 years (Amanna et al. 2007). However, there is a potentially negative consequence of the long-lived memory B cells and memory plasma cells, as antibodies directed against self-proteins can result in life-long autoimmune disease (Yoshida et al. 2010).

In one thoroughly studied developmental pathway, memory B cells and memory plasma cells are generated via somatic hypermutation of immunoglobulin genes and selection of high-affinity clones in the germinal center cycle that takes place in follicles of secondary lymphoid organs such as the spleen and lymph nodes (Griffiths et al. 1984; Berek et al. 1991; Jacob et al. 1991) (see germinal center cycle in Volume 1, Chap. 7). This developmental pathway depends on help provided by $CD4^+$ T follicular helper (T_{FH}) cells, which support the differentiation of antigen-specific B cells into memory B cells and plasma cells (Tangye et al. 2013; Griffiths et al. 1984; Yong et al. 2009; Grimbacher et al. 2003). There is a second pathway of memory B cell and memory plasma cell generation that is not dependent on the germinal center cycle, but that pathway(s) is not completely elucidated (Yoshida et al. 2010). See (Yoshida et al. 2010) for a review of memory B cells and memory plasma cells.

1.6.2.2 Memory T Cells

The generation of memory T cells is a major facet of immune protection. Antigen-specific naïve $CD4^+$ or $CD8^+$ T cells become activated following antigen exposure, and populations of the activated T cells undergo expansion and differentiation into effector T cells (Farber et al. 2014). There is general consensus that these expanded T cell populations constitute the precursors of antigen-specific, long-lived memory T cells which survive in multiple sites and participate in immune responses following re-exposure to pathogens (Farber et al. 2014; Remakus and Sigal 2013; Wherry and Ahmed 2004). There are three distinct phases of existence for memory cells: generation, homeostasis, and immunosenescence. In humans, memory T cells are generated primarily during infancy, youth and young adulthood (ages 0–20 years, (Farber et al. 2014; Cossarizza et al. 1996; Sathaliyawala et al. 2013; Saule et al. 2006). Homeostasis of memory T cells is maintained in humans through age 65, after which the process of immunosenescence commences (Cossarizza et al. 1996; Saule et al. 2006; Goronzy and Weyand 2013; Nikolich-Zugich and Rudd 2010) (see Volume 1, Chap. 5).

Memory T cells persist throughout adult life, and constitute the most abundant lymphocyte population in the body (Sathaliyawala et al. 2013; Kupper 2012). Levels of memory T cells in the blood markedly underestimates the total number of memory T cells in the body. In humans it has been estimated there are a total of 1, 2, 3 and 20×10^{10} T cells in lung, skin, intestine and lymphoid tissues, respectively (Clark et al. 2006; Purwar et al. 2011; Ganusov and De Boer 2007), therefore the peripheral blood T cell content of $5–10 \times 10^9$ cells represents only 2–2.5% of the total T cell population in the body (Ganusov and De Boer 2007).

Memory T cells are classically distinguished by expression of CD45RO and lack of expression of CD45RA (Sanders et al. 1988; Smith et al. 1986), and can be subcategorized by expression of CC-chemokine receptor 7 (CCR7) (Sallusto et al. 1999). $CCR7^+$ central memory T cells (T_{CM}) home to lymphoid tissues, while $CCR7^-$ effector memory T cells (T_{EM}) migrate to multiple tissue sites (Sallusto et al. 1999; Masopust et al. 2001; Reinhardt et al. 2001). Both T_{CM} and T_{EM} cells can respond to antigens, but T_{CM} cells have a higher proliferative capacity (Wang et al. 2012a; Fearon et al. 2006). A third category known as stem cell memory T cells (T_{SCM}) is identified by expression of FAS (CD95) and the memory-associated marker CD-122. T_{SCM} cells have the ability to differentiate into either T_{CM} and T_{EM} cells (Gattinoni et al. 2011; Gattinoni et al. 2012). A fourth tissue-resident memory T cell (T_{RM}) population is subdivided into a $CD4^+$ subset that resides in lung and bone marrow and a $CD8^+$ subset that resides in skin, vagina, intestine, lungs and brain (Turner et al. 2014; Herndler-Brandstetter et al. 2011; Clark et al. 2012; Liu et al. 2010; Mackay et al. 2012; Shin and Iwasaki 2012; Masopust et al. 2010; Wakim et al. 2010). The T_{RM} cells promote rapid local responses to viral infections.

The mechanisms by which memory T cell populations persist throughout the lifetime of the host are not completely known. It appears there are differences in the population maintenance processes for T_C versus T_H populations. Virus-specific $CD8^+$ T cells do not require antigenic stimulation or MHC molecules for their mainte-

nance, but are dependent on IL-15 for homeostasis and IL-7 for survival. By contrast, $CD4^+$ T cells require repeated TCR signaling and/or MHC class II molecules for homeostasis (Bushar et al. 2010; Kassiotis et al. 2002; Kassiotis et al. 2006). Human memory T cells have a half-life of only 1–12 months (Macallan et al. 2003; Macallan et al. 2004; De Boer and Perelson 2013; Vukmanovic-Stejic et al. 2006), thus it is clear that persistence of memory T cell populations does not involve continued survival of individual cells for a prolonged period. Memory T cells have less replicative potential than naïve T cells, and memory T cells have shorter telomeres than naïve T cells, which indicates the memory T cells have had a greater number of replicative cycles (Weng et al. 1995)**.** These observations suggest the circulating memory T cell population is maintained by continuous homeostatic turnover.

Development of immunological memory is not orchestrated entirely within the various cell populations of the adaptive immune system. Mesenchymal stromal cells contribute to, and regulate, immunological memory by organizing dedicated survival niches for plasma cells and memory T cells in the bone marrow and secondary lymphoid organs (Tokoyoda et al. 2010).

1.7 Immune Responses to Infectious Diseases

The immune response that has developed in large part to combat invading pathogens is accomplished through a coordinated response between the innate and adaptive immune responses. In large part the innate immune response is involved in blocking the entry or initial growth/colonization of microbes at normal physical barriers. These areas are the skin, lungs, respiratory and gastrointestinal tracts which have physical barriers (e.g., tight junctions at the skin surface), antimicrobial peptides and mucus at mucosal sites, innate immune cells and their products, specific responses (e.g., inflammation) which promote recruitment of adaptive immune cells, increased blood flow and various other mechanisms to favor the successful defense against the invading pathogen (Abbas and Lichtman 2015). The innate immune response provides early, rapid and non-specific responses while the adaptive immune response provides more specialized responses and an amnestic (memory) response to future challenge. Damage to the host can occur when infection remains due to the pathogen successfully evading immune responses or can also occur due to the host response to a microbe (e.g., tissue inflammation and damage, cytokine storm, etc.). There are numerous mechanisms by which pathogens attempt to evade these immune responses (Monack et al. 2004; Diacovich and Gorvel 2010).

Immunity to bacteria: Extracellular bacteria can induce inflammation and destroy tissues not only due to presence of the pathogen itself but also due to toxins produced by the pathogen. Immunity to extracellular bacteria occurs via several pathways, including complement activation, activation of phagocytes, and subsequent inflammatory (innate and adaptive) responses. Numerous innate immune receptors (TLRs, NLRs, etc.) are involved in recognition and the development of an immune response against bacteria (Diacovich and Gorvel 2010). Humoral immunity is a

significant protective mechanism which not only blocks and eliminates infection but also can neutralize toxins. APCs help promote T_H2 responses which enhance these humoral responses. Humoral antibodies, particularly IgG, IgM and IgA, can be directed against bacteria components and toxins. These antibodies can help neutralize, opsonize and phagocytose the pathogenic organisms and activate complement but do have the potential, when dysregulated, to cause antibody-mediated disease. One such example is post-streptococcal carditis and glomerulonephritis. Neutrophils and macrophages produce reactive oxygen species and lysosomal enzymes which serve to eradicate the infection but may also induce tissue damage. Inflammatory cytokines which play an important role against a pathogen may, when dysregulated or markedly induced, cause cytokine storms, septic shock and multi-systemic organ failure.

Immunity to intracellular bacteria is accomplished primarily by cell-mediated immunity. Neutrophils, macrophages and NK cells, $CD8^+$ CTLs and T_H1 $CD4^+$ T cells, T_H1 cytokines IL-12 and IFN-γ and cellular killing are important mechanisms for combatting and ultimately killing intracellular bacterial infections. Chronic antigen stimulation and failed attempts to resolve infection with intracellular bacteria can result in pathologic states such as granulomatous inflammation or persistent infections. Such persistent bacterial infections involve competing strategies by both pathogen and the host (Monack et al. 2004). Infection with *Mycobacterium tuberculosis* and *Helicobacter pylori* are two such examples. Additionally, dysregulated immune responses or the balance between T_H1 and T_H2 subsets can influence the outcome of various infections as can be seen in *Leishmania major* or *Mycobacterium leprae* infections (Abbas and Lichtman 2015).

Immunity to viruses: Viruses are intracellular pathogens and, as such, cell-mediated immune responses are critical in resolving viral infections (Rouse and Sehrawat 2010). Viruses infect cells via specific receptors, replicate intracellularly, and cause cytopathic effects and ultimate lysis. CTLs are an important defense against the viruses which reside persist in cells. Viruses can also cause latent infections where the virus can persist in infected cells, often for the life of the host. Such latent infections are particularly difficult to eradicate since viral proteins are often at low levels when the infection is quiescent and, thus, the immune system is often not stimulated or only minimally stimulated to target and kill infected cells. Type I interferons (IFN-α and IFN-β), which are induced by multiple pathways, are key cytokines involved in triggering appropriate immune responses against viral infections. Recognition of viral RNA and DNA by endosomal TLRs and activation of RIG-like receptors by viral RNA and STING pathway by viral DNA can all serve to promote effective anti-viral immunity (Rouse and Sehrawat 2010; Abbas and Lichtman 2015). Type I IFNs can prevent infection and also activate immune cells such as NK cells which can then kill infected cells.

Though cell-mediated immunity has a major involvement in anti-viral immunity, both humoral and cell-mediated immune responses are involved in protection against viruses. Specifically, antibodies produced by B cells can neutralize, block entry and protect against infection while $CD8^+$ CTLs can kill infected cells. Like all microbial infections, there is a balance between immune responses and viral eva-

sion and effective elimination of pathogens while limiting damage to the host (i.e., immunity versus immunopathology (Rouse and Sehrawat 2010)). Viruses (and other pathogens) can produce immunoregulatory compounds or provide other means which can directly or indirectly inhibit or dysregulate the immune response. Additionally, the host's response may of itself result in significant injury. For example, lymphocytic choriomeningitis virus (LCMV) is a non-cytopathic virus but the host's immune responses can result in meningitis.

Immunity to fungal infections also involves both innate and adaptive immune responses, and complex mechanisms are involved in pathogen-host interactions during fungal infection (Romani 2011). Cell-mediated immune responses are the primary mechanism by which fungal infections are resolved. T_H17 responses are strongly elicited during fungal infections with subsequent recruitment of neutrophils and monocytes to help destroy the function. C-type lectins (e.g., dectin-1), TLRs, NLRs and other innate immune signaling pathways can all play important roles in recognition and immune response to fungal infection. For example, dectin-1 is a receptor for fungal glucans on DCs and when stimulated, is a potent inducer of T_H17 responses (Abbas and Lichtman 2015). Antibodies to fungi can also develop but are less important than a strong cell-mediated immune response in eliminating infection. As seen in other infections, immune dysregulation can occur during fungal infections. For example, *Cryptococcus neoformans* can inhibit the production of pro-inflammatory cytokines, such as IL-12 and TNF-α, and stimulate IL-10 production which down-regulates macrophages, an important cell in eliminating infection (Abbas and Lichtman 2015). Fungal infections cause numerous diseases that are directly due to the agent, and also induce various immune-mediated diseases. The latter include respiratory asthma, various skin diseases (e.g., psoriasis), recurrent vulvovaginal or mucocutaneous candidiasis and inflammatory bowel disease (Romani 2011).

Immunity to parasites: Pathogenic metazoan and protozoan parasites are typically too large to be phagocytosed or have unique intracellular life cycles that shield them from immune recognition, thus there are complex immune responses to parasites. Protozoan parasites are single-cells organisms that include ciliates, amoebae and flagellates, while metaozoans are multicellular (e.g., helminth infections). *Giardia lamblia* (i.e., Giardia) and *Taenia solium* (pork tapeworm) are examples of protozoan and metaozoan parasites, respectively. Due to the multicellular nature and large size of helminths, the immune response is distinct. T_H2 cytokines (e.g., IL-4, IL-5, IL-13), antibodies including IgG1, IgG4 and IgE, and numerous immune cells (eosinophils, basophils, mast cells and alternatively activated macrophages) play important roles in eradicating infection. Since helminthic parasites are large, individual cells, direct lysis is relatively ineffective. IL-4, a key cytokine at promoting the necessary responses against helminth infections, not only induces T_H2 responses but can also have effects on various host cells to produce anti-microbial products. For example, IL-4 promotes differentiation of goblet cells, which increases mucus production, as well as the production of proteins with anti-helminth activities (e.g., resistin-like molecule-β; RELMβ). Peristalsis is often increased, which aids in

physical elimination of luminal helminths, and cytokines such as IL-9 and IL-18 increase mast cell functionality within the gut.

Both mucosal sites and non-mucosal sites can be infected by metazoan parasites. While reactions at mucosal sites may primarily function to exclude (e.g., prevent infection or colonization) of the parasite, non-mucosal sites typically must destroy the parasites and can do so via the action of basophils, eosinophils and antibody-mediated mechanisms (Allen and Maizels 2011).

Many protozoan parasites have evolved to survive intracellularly and have a complex life cycle with variable antigen expression so adaptive immune responses and traditional cell-mediated immune responses can be an important response to these parasites. Malaria caused by *Plasmodium* species is one such example, and numerous components of the immune response play a role in controlling this disease. Some important mechanisms by which these intracellular protozoal parasites are eradicated are various T_H2 responses, including the production of IgE antibodies. Eosinophils and mast cells also have important protective roles.

Parasites can result in dysregulation of immune response through several mechanisms. While T_H2 responses can be promoted, a persistent infection or the inability to remove the offending parasite can result in chronic antigenic stimulation and subsequent exhaustion. Alternatively activated macrophages, dysregulated APCs, induction of regulatory T cells and the induction of various regulatory immune responses either primarily or secondarily induced by the parasite can occur during infection (Maizels et al. 2009; Maizels and Yazdanbakhsh 2003).

1.8 Adverse or Nonspecific Immune Activation and Immune Dysregulation

1.8.1 Accidental or Incidental Immunologic Activation

Accidental or incidental activation of immunologic processes may result in substantial injury to the host. There are four major categories of this type of immune system activation: molecular mimicry, epitope spreading, bystander activation and cryptic antigen exposure. In **molecular mimicry** there is activation of cross-reactive T_H cells that recognize both microbial and self antigens, with resultant inflammatory response that results in host tissue injury (Blank et al. 2007). A prominent example is the cross-reactivity between streptococcal M protein and cardiac myosin, which results in rheumatic heart disease subsequent to group A streptococcal infection in humans (Cunningham 2012, 2014, 2016). **Epitope (determinant) spreading** is the development of immune responses to endogenous epitopes secondary to the release of self antigens from damaged tissues during a chronic autoimmune or inflammatory response (Vanderlugt and Miller 1996). This can result in immune-mediated damage to tissues that are distant from the site of the initial inflammation. **Bystander activation** of T_H cells is a nonspecific process whereby infiltrating T_H cells at a site

of inflammation includes a subpopulation of T cells that develop self-reactivity by TCR-dependent and -independent mechanisms. **Cryptic antigen exposure** results from the inflammation-mediated release of heretofore cryptic tissue antigens, with subsequent activation of T_H cells and associated inflammatory processes (Vanderlugt and Miller 2002).

Molecular mimicry and tissue cross-reactivity. Antibodies (and TCRs) recognize the three-dimensional profile of antigens rather than the chemical sequence, therefore, antibodies to one antigen may cross-react with an unrelated antigen that has a similar profile ('molecular mimicry'). Of course, antibodies to a specific peptide sequence will also react to the same peptide sequences if they are encountered in another tissue, even if the tissue is normal. It is common practice to describe this latter process as a form of 'molecular mimicry', though it seems more appropriate to consider these reactions to be a result of 'epitope homology'. The best-known pathologic process resulting from epitope molecular mimicry is the heart lesion ('rheumatic fever') that results from cross-reactivity between cardiac myosin and some species of S*treptococcus* bacteria (Cunningham 2014, 2016, 2012). Other potential examples of mimicry-associated diseases include chronic cardiomyopathy associated with *Trypanosoma cruzi* infection, Lyme arthritis associated with the OspA protein of *Borrelia burgdorferi* infection (Raveche et al. 2005; Steere et al. 2001; Trollmo et al. 2001), chronic myocarditis associated with coxsackievirus infection (Gauntt et al. 1995), and keratitis associated with herpes simplex virus-type 1 (Zhao et al. 1998). The suspicion of molecular mimicry and associated autoimmunity in these diseases is partially based on absence of the infectious agent in the lesions of the chronic disease process. While the evidence for molecular mimicry-associated autoimmunity is impressive, it is difficult to distinguish between molecular mimicry versus an alteration in local protein structures due to the previous presence of the infectious agent. These protein alterations may result in the creation of a new antigenic peptide or release of a cryptic antigen, either of which could provoke an immunologic response (Benoist and Mathis 2001). Alternatively, the inflammatory process associated with the infectious agents could alter the local population of professional or non-professional APCs, rendering them more active in the presentation of antigens to T cells (Benoist and Mathis 2001).

1.8.2 Heterologous Immunity

Homologous immunity, in which a host develops an immune response upon re-exposure to an antigen, is a critical element of immunological memory and traditional vaccine-induced immunity. Heterologous immunity, by contrast, refers to immunity that develops to a pathogen after the host has been exposed to a non-identical pathogen (Clark 2001; Selin et al. 1998; Welsh and Selin 2002). Heterologous immunity was identified early in the histology of immunology with the observation by Jenner and others that exposure of milkmaids to cowpox lesions on the udders of cows afforded some level of protection against human smallpox.

Heterologous immunity most commonly involves closely related pathogens, but on occasion may involve immunity to widely diverse pathogens. Heterologous immunity may be mediated by antigen-specific cross-reactive T cells, or in a less specific fashion by macrophages that are activated in areas of infection or inflammation. Heterologous immunity typically does not afford the same level of protection as homologous immunity, but heterologous immunity can be somewhat protective and of value to the host. Of course, the heterologous immune response is not necessarily positive and protective, since it is possible that the heterologous antigenic target is a normal tissue component.

1.8.3 Hypersensitivity Reactions Type I–V

See Volume 1, Chap. 7 for presentation of hypersensitivity reactions as they pertain to non-clinical toxicology studies. For additional information, see reviews and book chapters by Leach and Snyder (Snyder 2007; Snyder 2012; Leach 2013; Leach et al. 2014).

1.8.4 Immune Dysregulation in Cancer

Immune dysregulation contributes to the development of numerous autoimmune diseases, to the point that a discussion of the immune contributors and pathogenesis of autoimmune disease is beyond the scope of this chapter. Many of those discussions are included in the various organ system chapters. However, as autoimmune and allergic diseases continue to increase, an understanding of the various contributors and heterogeneity in pathogenesis of these diseases is of increasing interest and has important implications for developing new therapies (Cho and Feldman 2015; O'Shea et al. 2002; Eisenberg 2003; Lucas and Lenardo 2015).

One area of immune dysregulation of increasing interest in the drug development arena is the immune dysregulation that occurs during cancer and how this impacts and can be harnessed in the design of new therapies. The immune system is designed to provide immunosurveillance to protect against the development of neoplasms and provide immune recognition and elimination (i.e., destruction) of neoplastic cells that develop (Dunn et al. 2002). Both innate and adaptive immune responses play important roles in mediating the removal of neoplastic cells (Gajewski et al. 2013). Cell-mediated responses (i.e., NK cells and CTLs) are particularly important in recognizing and destroying neoplastic cells (Abbas and Lichtman 2015). $CD8^+$ T cell responses may require cross-presentation of tumor antigens by DCs to be maximally activated. This is accomplished most commonly by host APCs ingesting tumor cells (or their antigens) and presenting peptides derived from those ingested cells or cell fragments to CTLs to induce an anti-tumor response. Tumor antigens (e.g., molecules or products unique to tumor cells, products that are silent in normal

tissues or present only within immune privileged sites, or other factors that identify the tumor as altered self) can be recognized by immune cells, thus targeting tumor cells for destruction. However, some tumor cells are able to evade these anti-tumor immune responses. Tumor cell variants, through mutations resulting in altered antigen expression and other immune avoidance mechanisms, escape attack and removal by the immune system and continue development to form neoplasms (Abbas and Lichtman 2015; Chew et al. 2012; Kim et al. 2007)). Neoplastic cells and tumors can also contribute to further immunologic escape through development of multiple mechanisms which suppress the anti-tumor immune response. In general, cancer is considered a state of immunosuppression (or immunomodulation) which prevents immune recognition and allows tumors to progress (Zou 2005). Regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages are potent immunoregulatory immune cells which limit robust anti-tumor immune responses, thus have may have a detrimental effect on overall host well-being (Abbas and Lichtman 2015). Cancer immunotherapy, a relatively new field that was considered a "breakthrough of the year" in 2013, uses the immune system to combat cancer (Mellman et al. 2011; Topalian et al. 2011; Couzin-Frankel 2013). Different than traditional chemotherapies that target and kill tumor cells, immunotherapy aims to stimulate the body's own anti-tumor immunity in a highly specific manner, or combat tumor-mediated immune suppression (Motz and Coukos 2013). There are numerous therapies that are being explored and developed that capitalize on cancer immunotherapy. One approach is to stimulate anti-tumor immune responses through vaccination strategies and/or cellular therapies that target immune responses against the tumor. Adoptive cell therapy with either tumor-antigen loaded DCs or unique T cells expressing chimeric antigen receptors (CARs) have shown success in treating patients, as have anti-tumor antibodies that are specific against a given target tumor. Cytokine therapies that can stimulate the proliferation and differentiation of T and NK cells can provide benefit (e.g., IL-2), as does non-specific stimulation of the immune system with various immunostimulants or adjuvants. More recently, there is keen interest in blocking the inhibitory pathways that are mounted by some tumors. Checkpoint blockade is one such therapy where tumor-associated limitation of T cell responses (e.g., via CTLA-4 and PD-1 production by tumor cells) are pharmaceutically inhibited and essentially release the brake on natural anti-tumor immunity (Sharma and Allison 2015).

The contribution of immune dysregulation in cancer has been well established and therapies designed to modify immune responses are increasing each year. Additionally, there are numerous other diseases where inflammation, immune dysregulation and the immune system contribute to disease pathogenesis, progression or failure of resolution (Kuek et al. 2007; Pawelec et al. 2014). Given the large number of potential individuals that could be impacted by developing appropriate immunotherapies or immunomodulatory therapies, the number of biologic and small molecules therapies targeting immune responses is likely to increase (Kuek et al. 2007; Sozzani et al. 2014; Laveti et al. 2013). This fact highlights the importance of understanding both basic immunology concepts and how dysregulated immune responses contribute to the pathogenesis of various diseases.

1.8.5 Immunosenescence

Aging has a deleterious effect on multiple components of the mammalian immune system. The most spectacular change is thymic involution, which commences near the age of puberty and progresses to the point that only small remnants of thymic tissue are present in the middle-aged to aged adult. Spontaneous age-related thymic involution poses problems in the histopathological evaluation of toxicology studies, as the age-related changes must be distinguished from xenobiotic- or stress-associated immunomodulation. Less pronounced age-related histologic changes in other organs of the immune system include reduced cellularity and increased adipocyte populations in the bone marrow, reduction in the number and cellularity of lymphoid follicles in secondary lymphoid organs (spleen, lymph nodes and MALT), and a reduction in the number of follicular germinal centers. Immune system organs of aged animals also have a number of incidental histologic alterations that are not directly related to immunological functioning, but may complicate detection and interpretation of xenobiotic-associated immunomodulation. Prominent among these incidental changes are pigment accumulation in the spleen and lymph nodes, sinus dilatation or cystic degeneration of lymph nodes, formation of epithelial nests and tubules in the thymic remnant (rats), and accumulations of macrophages in various locations.

The overall effect of aging is a reduction in immunological function, but the age-related changes are not necessarily associated with a decrease in the number of immune system cells. In many cases the age-related immunological deficit is due to reduced or dysregulated immune functions. Virtually all cellular components of the innate and adaptive immune systems are affected.

Effects on the innate immune system: Age-related changes in macrophages include reduced phagocytic ability, cytokine and chemokine production, expression of MHC class II, and expression of co-stimulatory molecules such as CD80, thus there is a reduction in macrophage function in both the innate and adaptive immune systems. Age-related changes in dendritic cells include reduced migration, pinocytosis, phagocytosis and stimulation of T cell and B cell responses. Aging effects on NK cell function involves a paradoxical increase in the number of circulating NK cells which is offset by reduced NK cell function, resulting in an overall reduction in NK cell-related immunological functions. NK cell-mediated killing of cells with absent or atypical MHC expression is thought to be a major component of tumor immunity, thus the age-related decrement in NK cell activity may contribute to the increased incidence of neoplasia in older subjects.

Effects on the adaptive immune system: Age-related thymic involution is associated with a dramatic reduction in the number of naïve T cells exiting the thymus. Subsequent extrathymic T cell generation, which occurs largely in the intestine and biliary tract, lacks the effective selective processes that exist in the thymus, thus the resultant extra-thymically generated T cells have a greater propensity for autoreactivity. The major T cell co-stimulatory pathways (CD28/B7 and CD40/CD40L) become dysfunctional with aging. The numerical ratio of $CD4^+$ to $CD8^+$ T cells

declines with aging. In addition, multiple processes conspire to result in an age-associated increase in the number of T cells that lack functional CD28 molecules, thus are unable to participate in the CD28/B-7 conjugate that is an early event in T cell activation. In humans, nonhuman primates and probably other species, there is an age-related increase in the number of T cells that are committed to responses to cryptic viral antigens such as cytomegalovirus, Epstein–Barr virus and herpes simplex virus, the reducing the population of T cells that are available to respond to other pathogens. Age-related effects on T_H cells result in deficits in B cell function, but there are also direct age-related effects on B cells. These include decreased production of B cell precursors by the bone marrow, reduction in the B cell immunological repertoire, and accumulation of memory B cells at the expense of effector or naïve B cells. The combination of changes in cell populations and functions results in reduced germinal center activity in secondary lymphoid organs, with attendant reduction in the production of high-affinity immunoglobulins and generation of highly specific memory B cells. These latter factors are involved in the reduced efficacy of vaccines in the elderly human population.

Aging results in a generalized proinflammatory status in the host ('inflamm-aging')(Franceschi et al. 2000), which predisposes the host to inflammatory disease processes and exacerbates concurrent disease processes such as atherosclerosis, neurodegeneration and osteoporosis. The proinflammatory status results from a number of age-related changes in cell populations as well as signaling and effector molecules. Macrophages and dendritic cells of aged subjects have reduced ability to phagocytize the cellular debris that results from apoptosis of effete cells, resulting in accumulation of cellular debris that may provoke an inflammatory response. Neutrophils of aged subjects have reduced phagocytic ability as well as reduction in the oxidative burst that is involved in microbial killing. Circulating effete neutrophils normally return to the bone marrow by a process similar to the chemotactic process that relocate neutrophils to sites of inflammation. Age-related reduction in neutrophil chemotactic ability has the dual detrimental effects of (1) reduced neutrophil effectiveness in combating infections and (2) reduced ability of effete neutrophils to return to the bone marrow for normal destruction. The resultant increased population of circulating neutrophils with reduced functional capacity contributes to the general pro-inflammatory status, but with the paradoxical effect of reduced host protection against infection.

See Volume 1, Chap. 5 for more detailed information on immunosenescence.

1.8.6 Cytokine Storm

"Cytokine storm" refers to a cascade of proinflammatory cytokines, which results in numerous harmful effects that include fever, pain and organ system failure due to hypotension (Suntharalingam et al. 2006). Cytokine storm is best exemplified by the 'Te Genero incident', which involved catastrophic results in six clinically healthy human volunteers in a phase 1 clinical trial of TGN1412 (Suntharalingam et al.

2006; Stebbings et al. 2007). Similar cytokine releases have been seen with other candidate drugs, including rituximab (Winkler et al. 1999; Gaston et al. 1991; Wing et al. 1996).

A summary of T cell activation is helpful in understanding the immunologic processes involved in the Te Genero incident. Activation of B cells and subsequent antibody production involves a sequence of steps involving T_H cells and two co-stimulatory molecules and their ligands: CD40/CD40L and B7/CD28-CTLA-4. The co-stimulatory molecule CD40 is expressed on all B cells, and its ligand (CD40L) is expressed on the T_H cell upon activation by specific antigen in MHC-II context. Interaction between B7-1/B7-2 (CD80/86) with CD28 or CTLA-4 is the second important step in the T cell/B cell interaction that results in the humoral immune response. The co-stimulatory molecule B7 (CD80/86) is expressed on dendritic cells, activated macrophages and activated B cells, which serve as antigen-presenting cells (APCs). The co-stimulatory molecule CD28, which has moderate affinity for B7, and CTLA-4, which has high affinity for B7, is expressed on T_H cells. The B7/CD28 interaction has a positive effect on the humoral immune response, while B7/CTLA-4 interaction has a damping effect on the humoral response and helps to control auto-reactivity (Rudd and Schneider 2003; Rudd et al. 2009; Chikuma and Bluestone 2003; Greenwald et al. 2005). Pharmacological influence on these basic processes has the potential to generally augment, or generally suppress, humoral immunity. However, intervention in these processes, which are at the heart of humoral immunoreactivity, carries the potential for a major negative impact on the host.

The Te Genero incident involved a phase I clinical trial in which six healthy male volunteers received TGN1412, a novel superagonist anti-CD28 monoclonal antibody that directly stimulates T cells without involvement of a co-stimulatory molecule. Two additional volunteers received placebo, and developed no clinical complications. Within 90 min of receiving a single intravenous dose of TGN1412, all six volunteers developed a systemic inflammatory response that included headache, lumbar myalgia, nausea, diarrhea, erythema, vasodilation, and hypotension. The patients became critically ill and developed multiorgan failure within 12–16 h, exhibiting lung injury and pulmonary infiltrates, renal failure, and disseminated intravascular coagulation. Two patients developed cardiovascular shock and acute respiratory distress syndrome, and required intensive care for several days. All six patients survived, though there were significant residual medical effects.

Prior to the Te Genero incident it was believed that superagonistic CD28-specific monoclonal antibodies (mAb) did not trigger a toxic cytokine storm (Rodriguez-Palmero et al. 2006; Beyersdorf et al. 2005). The Te Genero incident provoked immediate responses from regulatory agencies and the pharmaceutical industry due to general concerns that standard non-clinical toxicology studies may be inadequate for detection of potentially serious immunomodulation in humans. Subsequent investigation revealed a number of contributory factors in the preclinical studies of TGN1412. *In vitro* studies performed prior to the phase I clinical trial did not predict *in vivo* toxicity because the *in vitro* studies did not involve presentation of the TGN1412 mAb to white blood cells (WBC) in a manner that mimics its *in vivo*

presentation (Stebbings et al. 2007). Simply adding TGN1412 to WBC in aqueous solution failed to stimulate cytokine release (Hanke 2006), apparently because binding of TGN1412 to cell surfaces is a prerequisite for triggering cytokine release (Stebbings et al. 2007). In addition, WBC from cynomolgus macaques, one species used in the non-clinical toxicology studies of TGN1412, do not respond to TGN1412 in the same way as human WBCs respond. In essence, TGN1412 is superagonistic in humans but not in cynomolgus monkeys (Stebbings et al. 2007), but the reason for this difference is unclear. Human and cynomolgus macaque CD28 extracellular domains are 100% identical, but this may not preclude functional differences (Ohresser et al. 2006). It is known that three transmembrane residues of macaque CD28 are different from those of humans, which could alter the associations of CD28 with intracellular molecular partners (Kenter and Cohen 2006).

In the laboratory rat, which was the other species used for non-clinical toxicology testing of TGN14122, CD28 superagonists cause an expansion of regulatory T cells (Beyersdorf et al. 2006b; Beyersdorf et al. 2006a; Lin and Hunig 2003). Therefore, superagonist stimulation of CD28 by TGN1412 would result in down-regulation of the immune response.

To summarize, the *in vitro* studies of TGN 1412 did not involve clinically relevant presentation of the test article to white blood cells, and TGN1412 does not function as a CD28 superagonist in either of the two laboratory animal species (rat and cynomolgus macaque) that are commonly used in *in vivo* non-clinical toxicology studies. Subsequent studies have shown the dose of TGN1412 given to the human volunteers was close to the maximum immunostimulatory dose (Stebbings et al. 2007).

The consensus wisdom derived from the Te Genero incident is that the design of preclinical studies for immunologically active biopharmaceutical products cannot be limited to standard protocols that are applied with a ‘one-size-fits-all’ approach. Design of these studies must involve investigators who are thoroughly familiar with immunology, immunotoxicology and immunopathology, and must involve very close communication with developmental scientists who have a thorough understanding of the immunobiological activity of the test molecule. Particularly careful attention must be given to the relevance of animal species used for the non-clinical toxicology studies, as well as the precise clinical relevance of any *in vitro* studies.

While the Te Genero incident is considered a classic example of cytokine storm, it should be noted that many of the clinical observations in the ill-fated Phase I clinical trial suggested concurrent eicosanoid storm, as presented below.

1.8.7 Eicosanoid Storm

Eicosanoids are locally active signaling lipids derived from arachidonic acid (20 carbon) and related polyunsaturated fatty acids (PUFAs) (Funk 2001; Buczynski et al. 2009). A related group of molecules known as docosanoids are signaling molecules derived from docosahexaenoic acid (22 carbons). Eicosanoids are derived from the oxidation of arachidonic acid and related PUFAs by cyclooxygenase

(COX) (Smith et al. 2000), lipoxygenase (LOX)(Kuhn and O'Donnell 2006), and cytochrome P450 (CYP) enzymes, or by non-enzymatic free radical mechanisms (Dennis and Norris 2015). Eicosanoids derived from the COX pathway are involved in a broad array of signaling pathways, but those derived from the 5-LOX pathway are more specifically targeted to promote bronchoconstriction (Samuelsson 1983) and leukocyte migration to sites of tissue damage (Lammermann et al. 2013). Eicosanoids have historically been known for their pro-inflammatory actions, but there is substantial evidence for the involvement of eicosanoids in resolution of inflammation (Serhan 2014).

Blocking the action of classical eicosanoids such as prostaglands and leukotrienes by the use of nonsteroidal anti-inflammatory agents (NSAIDs) is a long-standing strategy in relieving some components of inflammation. Some of the agonists and receptors that initiate the inflammation-related cytokine storm also initiate an eicosanoid storm (Gross et al. 2011; Latz et al. 2013; Norris et al. 2014). The nature of the eicosanoid storm induced in individual cell populations varies with the tissue in which the cells reside. For example, the constellation of prostaglandins produced by macrophages in response to engagement of TLR is variable, depending on the tissue from which the macrophages were isolated (Norris et al. 2011). There are also indications that age, diet and individual genetic variation impact on the formation and activity of eicosanoids.

The possibility of eicosanoid-associated immunomodulation is a significant concern to toxicologists and toxicologic pathologists. Life-threatening situations such as toxic shock syndrome, though commonly known as a 'cytokine storm', often include an eicosanoid storm that contributes substantially to the clinical presentation (von Moltke et al. 2012). Altered resistance to pathogens or commensal organisms is a common manifestation of immunomodulation following xenobiotic exposure, and it is clear that altered eicosanoid milieu as well as altered cytokine milieu may be involved in the pathogenesis of the immunomodulation. As a further complication, altered expression and activity of CYP enzymes, which is common following administration of xenobiotics, could have downstream effects on the formation of eicosanoids, with resultant positive or negative effects on immune responses.

See (Dennis and Norris 2015) for a review of eicosanoid interactions in infection and inflammation.

1.9 Major Ancillary Concepts and Processes

1.9.1 Leukocyte Emigration and Chemotaxis

Leukocyte migration from the bloodstream into peripheral tissues is an important step in the initiation and perpetuation of the inflammatory response. This complex process involves multiple signaling and effector molecules, and is subject to a number of checks and balances, thus includes many potential targets for pharmacological intervention in the inflammatory process. A thorough understanding of this basic

pathobiological process is critical to the practice of general toxicology and toxicologic pathology.

When a fluid mixed with particles flows in a tubular structure, the particles tend to be concentrated in the central aspect of the fluid stream unless the laminar flow is disrupted, in which case the particles become dispersed throughout the liquid and come into contact with the inner surface of the tubular conduit. This principle applies to the laminar flow of blood that contains leukocytes, which are particles being carried in a stream of plasma fluid. In postcapillary venules the laminar flow of the blood is disrupted and leukocytes come into contact with the endothelial cells lining the venules. (A similar disruption in laminar flow occurs as blood enters hepatic sinusoids). If an inflammatory reaction is developing in the nearby tissue, signaling molecules from the site of inflammation cause up-regulation of low affinity binding molecules (selectins) on the luminal surface of endothelial cells that line the post-capillary venules. Expression of some of the selectins is a post-translational event, thus involves a time delay in expression, but pre-formed selectins contained within the Weibel–Palade bodies of endothelial cells are available for immediate expression upon receipt of an appropriate signal. Sequential selectin-mediated binding and release between endothelial cells and leukocytes result in slow movement of leukocytes along the endothelial surface, as process known as ‘rolling’ of leukocytes. Endothelial cells that have been activated by pro-inflammatory cytokines for several hours start to express E-selectin, which further slows the movement of leukocytes in a step known as ‘slow rolling’. The microscopic manifestation of this process is known to histopathologists as ‘pavementing’ of leukocytes, which is commonly observed in blood vessels near sites of inflammation. Following the low-affinity, selectin-mediated rolling step, leukocytes become more firmly bound to endothelial cells via interactions between high-affinity integrin molecules on leukocytes and ligands such as ICAM-1, ICAM-2 and VCAM-1 on the luminal surface of endothelial cells. Antibodies or xenobiotics that block selectin function serve to inhibit the leukocyte rolling behaviour, as does genetic deletion of selectin molecules. Molecules or genetic deletions that interfere with integrin function prevent firm adhesion of leukocytes to the luminal surface of endothelial cells. Interference with either of these steps impedes development of the inflammatory reaction, which may be a pharmacologically desirable end-point.

Leukocytes then exit the postcapillary venule by the process of diapedesis, which is facilitated by platelet-endothelial cell adhesion molecule-1 (PECAM-1). The transendothelial migration occurs primarily at endothelial cell borders, thus is termed ‘paracellular transmigration’, though ‘transcellular migration’ directly across endothelial cells is known to occur. Once the leukocytes exit into the extravascular space, they are guided to the site of inflammation by chemokines and other signaling molecules. Different leukocyte populations emigrate at different times in the development of the inflammatory reaction, a feature which has historically allowed histopathologists to subclassify inflammatory reactions as ‘acute’ (neutrophil predominance in the infiltrating cell population), ‘subacute’ (lymphocyte predominance), or ‘chronic’ (lymphocyte and macrophage predominance, typically with reactive fibroplasia). In addition to the chemokines and other signaling mole-

cules that guide leukocytes to the site of inflammation, it has been shown that neutrophils of the first emigration wave leave cellular fragments called 'neutrophil trails' in their wake, and those fragments guide subsequent waves of lymphocyte emigration (Lim et al. 2015).

The intercellular junction between adjacent endothelial cells is not merely an empty channel through which leukocytes emigrate into surrounding tissues. The lateral borders of endothelial cells have regions known as lateral border recycling compartments (LBRCs) that exhibit continuous movement and recycling of components between the vascular lumen and the extravascular space. Nearly 30% of the endothelial cell's PECAM is in the LBRC region, and the PECAM recycles with a half time of about 10 min (Mamdouh et al. 2003). The LBRC thus provides a continuously moving escalator that facilitates movement of activated leukocytes from blood vessels into the extracellular space.

Additional inflammation-related alterations in endothelial cells facilitate the emigration of leukocytes. Cross-linking of VCAM-1 and ICAM-1 on endothelial cells stimulates the release of free calcium ions, which activates myosin light change kinase (MLCK) (Huang et al. 1993). The activation of MLCK leads to actin-myosin fiber contraction, which helps to separate endothelial cells (Hixenbaugh et al. 1997). Stimulation of ICAM-1 also leads to phosphorylation of VE-cadherin, which is required for adherens junction disassembly (Turowski et al. 2008), and stimulates an increase in reactive oxygen species that cause loosening of adherens junctions (Cook-Mills et al. 2004).

Multiple reviews provide in-depth insight into the process of leukocyte emigration (Muller 2013, 2011).

1.9.2 Programmed Cell Death and Immunity

Cell death and inflammation have been known as concurrent events for over 100 years, but the molecular basis for the association has become apparent only in the past few decades. The definition of apoptosis as a mechanism of programmed cell death in the 1970s (Kerr et al. 1972) was a first step, followed in the 1990s by the proposals of the Pattern Recognition Theory (Janeway 1989) and the Danger Model (Matzinger 1994) that proceeded to identification of pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-1-like receptors (RLRs) (Medzhitov 2009; Martinon et al. 2009; Bergsbaken et al. 2009). Receptor recognition of the molecular patterns of organisms is highly context-sensitive, with similar molecular patterns being treated as 'normal' or pathogenic depending on location of the organisms and existence of additional signals of 'danger'. Whether an inflammatory response to cell death is generated is probably more dependent on the context of the event than the necrosis versus apoptosis death pathway of the event (Silke et al. 2015).

A wide variety of cellular insults result in the explosive form of cell death known as necrosis, which presents in various microscopic forms that commonly include

some element of inflammation. By contrast, programmed cell death (PCD) is a more orderly process whereby cells are shut down and removed, most commonly without provoking an inflammatory reaction. This broad-brush categorization of the features of necrosis versus PCD have come into question with the discovery that some forms of PCD result in release of inflammatory mediators, as presented below. PCD can represent a form of 'cellular altruism' in which individual cells are sacrificed for the overall benefit of the host, thus constituting a defense mechanism. PCD of infected cells can curtail microbial infection, thus sparing adjacent cells. Additionally, PCD can warn the host of impending danger via release of alert and danger signals. There are a number of forms of PCD (Galluzzi et al. 2012), but apoptosis, pyroptosis, and necroptosis have the most significant interactions with host defense.

Apoptosis is part of the normal life cycle of many tissues, and is triggered by extrinsic or intrinsic pathways. The extrinsic pathway is initialized by binding of extracellular ligands to transmembrane receptors, which leads to activation of caspase 8. Caspase 8 cleaves caspase 3 either directly or indirectly, leading to mitochondrial outer membrane permeabilization (Tait and Green 2010). The intrinsic pathway is initiated by DNA damage, microbial infection and development signals, and culminates in mitochondrial outer membrane permeabilization, release of cytochrome c into the cytoplasm, and assembly of an apoptosome composed of cytochrome c, apoptotic protease activating factor 1 (APAF1), and procaspase 9 (Tait and Green 2013).

Pyroptosis is an inflammatory form of PCD that results from activation of caspase 1 by a cytosolic inflammasome complex, which results in the release of proinflammatory cytokines such as IL-1β and IL-18. In contrast to apoptosis, pyroptosis does not involve caspase-1-mediated cleavage of downstream caspase molecules (Bergsbaken et al. 2009). Pyroptosis can occur in the absence of caspase 1 expression, due to the redundant role of caspase 11 (Kamens et al. 1995; Wang et al. 1996). Caspase 11 has two important features relative to host defense: (1) it is up-regulated by exposure to lipopolysaccharide (LPS) or tissue injury and (2) it is an upstream regulator of caspase 1. These features bring caspase 11 and pyroptosis into center stage with regard to septic shock associated with enteric pathogens. Caspase 11 has a central role in mediating LPS lethality in mice (Hagar et al. 2013), thus LPS exposure is not lethal in *Casp11* $^{-/-}$ mice. Pro-caspase 11 expression is induced upon infection by Gram-negative bacteria, regardless of the virulence of the microbes (Gurung et al. 2012; Rathinam et al. 2012), and pyroptosis is induced via pathways that apparently do not require the inflammasome of the canonical pathway. There is evidence that human caspases 4 and 5 serve as cytosolic receptors for intracellular LPS (Shi et al. 2014). These pathways are being investigated as potential sepsis therapy (Blander 2014).

Necroptosis is triggered by death receptor or TLR signaling. It is initiated by phosphorylation of receptor-interacting kinase 1 (RIPK1) by RIPK3. Activated RIPK1 in turn phosphorylates RIPK3, forming a RIPK1/RIPK3 complex called a 'necrosome'. Downstream effector molecules include the pseudokinase mixed

lineage kinase domain-like protein (MLKL), which is phosphorylated by RIPK3 (Sun et al. 2012; Zhao et al. 2012) and the mitochondrial serine/threonine phosphatase PGAM5 (Wang et al. 2012b). Phosphorylated MLKL oligomerizes and binds phosphatidylinositol phosphates and cardiolipin, thereafter translocating to the plasma membrane where it functions as a pore (Dondelinger et al. 2013; Wang et al. 2014a) or an ion channel regulator (Chen et al. 2014; Cai et al. 2014). Unlike apoptosis, necroptosis proceeds in the absence of mitochondrial involvement.

Programmed cell death is either tolerogenic or immunogenic depending on the specific form of cell death (Green et al. 2009). Pyroptosis and necroptosis result in rupture of cells, with release of cytosolic contents that include ATP, high mobility group protein B1 (HMGB1), pro-IL1α, and IL-33 (Kaczmarek et al. 2013), thus are immunogenic and pro-inflammatory. By contrast, apoptosis is immunologically silent in most circumstances. Apoptosis results in packaging of cellular contents into membrane-bound apoptotic bodies which shield the cellular debris from detection by PRRs or other elements of the immune system. The apoptotic bodies are phagocytized by macrophages by a process known as efferocytosis (Green et al. 2016), after which the phagocytized material is degraded via the phagosome/lysosome system. The apoptosis disposal system must be particularly robust in organs such as the thymus, in which more than 95% of developing cortical thymocytes undergo apoptosis due to positive and negative selection processes. Apoptosis is also particularly active during fetal and early postnatal organ development, during which major restructuring and resorption of cellular elements takes place. For example, 70% of the neurons present in the brain of a rat pup at postnatal day 7 are removed by apoptosis prior to postnatal day 14 (Bandeira et al. 2009). Immunological silence is necessary in these processes because an associated inflammatory or immunological reaction would be a hindrance to organ development and host survival.

See (Blander 2014) for a review of PCD involvement in immunity.

1.9.3 Autophagy and Immunity

Autophagy is a fundamental process by which intracytoplasmic cellular components are delivered to lysosomes for degradation and recycling (Yang and Klionsky 2010; Klionsky and Emr 2000). This function is centered on the autophagosome, which is formed from membrane sources such as the endoplasmic reticulum (Tooze and Yoshimori 2010), the endosome system (Puri et al. 2013), and phospholipid precursors (Dupont et al. 2014). Autophagy is thought to be an evolutionarily ancient defense mechanism that eukaryotic cells employed against invading microorganisms (Deretic et al. 2015). Starvation is an important signal in the initiation of autophagy, and is known to increase the level of autophagy-dependent killing of intracellular pathogens (Gutierrez et al. 2004; Tattoli et al. 2012).

Autophagy was initially known as a process whereby cytoplasmic organelles and protein complexes were sequestered into membrane-bound autophagosomes, which

fused with lysosomes containing lytic enzymes that degrade the organelles and proteins. This form of autophagy is now known as macroautophagy. In a second form of autophagy, known as microautophagy, cytoplasmic constituents bud directly into lysosomes. A third form of autophagy, known as chaperon-mediated autophagy (CMA), involves carrier-mediated transport of cytoplasmic molecular constituents from the cytoplasm into lysosomes (Bejarano and Cuervo 2010; Mizushima et al. 2008; Cuervo 2004; Fig. 1.7).

Though autophagy was originally described as a self-digestive process to remove intrinsic cellular elements, it is now clear that extrinsic pathogens may be cleared from the cytoplasm via autophagy (Andrade et al. 2006; Ling et al. 2006; Amano et al. 2006; Deretic 2006; Bradfute et al. 2013). These processes are controlled by autophagy-related gene-controlled (ATG) elements that have been characterized in yeast and mammals.

Downstream events subsequent to autophagic sequestration and digestion of intrinsic or extrinsic particles and molecules can have multiple effects on innate and adaptive immunity (Deretic 2005; Schmid et al. 2006; Deretic 2006). Prominent among these downstream events are the induction of type I interferons via TLR7-related signaling pathways in innate immunity (Lee et al. 2007; Lee et al. 2010), and feeding of antigenic peptides into MHC class II molecules in adaptive immunity (Levine and Deretic 2007; Deretic et al. 2013). Autophagy also has broad-spectrum effects on innate and adaptive immunity by downregulation of NF-κB signaling (Deretic et al. 2015).

Autophagy involvement in innate immunity: Autophagy is involved in both pro- and anti-inflammatory processes. In the pro-inflammatory role, autophagy delivers cytosolic pathogen-associated molecular patterns (PAMPs) for recognition by pattern recognition receptors (PRRs) such as TLRs in endosomes, which, for example, allows detection of viral replication and subsequent production of type I interferons by plasmacytoid dendritic cells (Lee et al. 2007). However, these same processes may contribute to autoimmune diseases if autophagy augments the PAMP/PRR responses to normal cellular constituents (Henault et al. 2012; Chaturvedi et al. 2008). Autophagy prevents unscheduled or excessive inflammasome activation but, once the inflammasome is established, autophagy contributes to inflammasome function. Autophagy serves to suppress inflammation by eliminating key functional components of the inflammasome as well as reducing the level of agonists that stimulate formation of the inflammasome. However, once the inflammasome is activated, autophagy contributes to the unconventional secretion of IL-1β as well as IL-18 and HMGB1, thus having a pro-inflammatory effect (Dupont et al. 2011; Ohman et al. 2014). On the opposing front, there is evidence that autophagy is involved in the degradation of pro-IL-1β (Harris et al. 2011), suggesting that the basic process of autophagy is involved in fine-tuning inflammatory responses mediated via the inflammasome.

Autophagy involvement in adaptive immunity: Autophagy has numerous effects on adaptive immunity, including effects on hematopoietic stem cells, T cells, memory B cells and plasma cells. It has a protective function in maintaining popula-

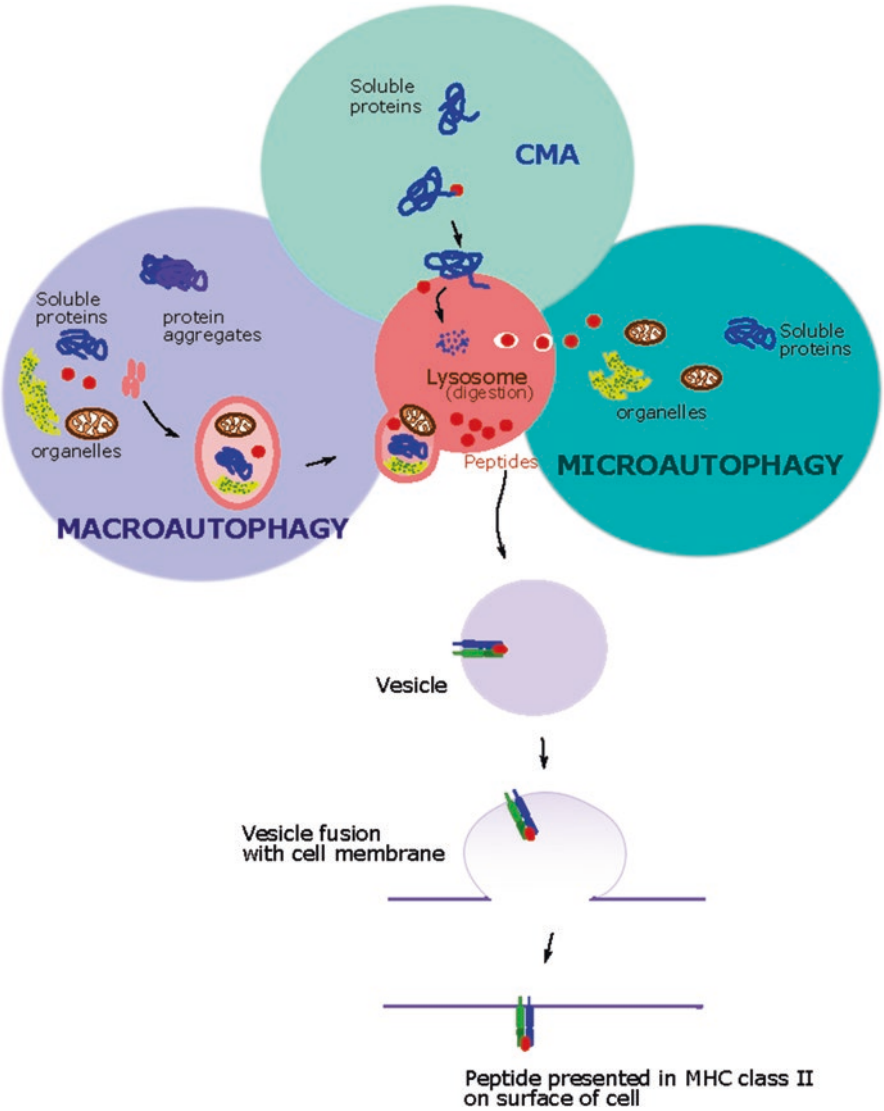

Fig. 1.7 Autophagy involvement in immunity. Three subcategories of autophagy feed into a common pathway whereby antigenic peptides are presented in MHC class II context on the cell surface. See text Sect. 1.9.3 and associated references for additional details. CMA = chaperon-mediated autophagy

tions of self-renewing hematopoietic stem cells, and influences the development, repertoire selection, maturation, homeostasis, function and polarization of T cells (Deretic et al. 2015). Autophagy delivers cytosolic proteins to antigen-processing compartments (Nedjic et al. 2008; Schmid et al. 2007; Blanchet et al. 2010; Jin et al.

2014), and is involved in the processing of extracellular particulate antigens that are assimilated via phagocytosis (Lee et al. 2010), the end result being presentation of antigenic peptides in the context of MHC class II.

Autophagy is involved in the direct elimination of microbial pathogens by two processes: xenophagy and microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP) (Henault et al. 2012; Sanjuan et al. 2007). Xenophagy is the sequestration of cytosolic microbes into membrane-bound autophagosomes (Levine et al. 2011), while LAP is the degradation process initiated by engulfment of extracellular pathogens via phagocytosis. In the absence of LAP, phagocytized microbes are protected by the membranes of the phagocytic vacuole and continue to exist within the cell. Xenophagy and LAP are guided by a subclass of PRRs known as sequestosome 1/p62-like receptors (SLRs) (Deretic et al. 2013). Failures in autophagic processes appear to be involved in chronic diseases such as tuberculosis and Crohn's disease (Deretic et al. 2015; Travassos et al. 2010).

There is evidence of genetic predisposition to some autophagy-related disease processes. For example, the human intestinal microbe *Bacteroides fragilis* produces immunomodulatory molecules that are released via bacterial outer membrane vesicles (OMVs. The OMVs trigger an ATG16L1- and NOD2-dependent noncanonical autophagy pathway in dendritic cells, which in turn induces Treg cells that protect against colitis (Chu et al. 2016). The OMVs protect mice from *B. fragilis*-associated colitis only if *Atg161I* and *Nod2* genes are functional in the mice (Chu et al. 2016). There is evidence the experimental system in mice is operative in humans, as the human orthologs of the *Atg161I* and *Nod2* genes are known to be involved in the predisposition to inflammatory bowel disease (Fujita et al. 2009; Henckaerts et al. 2011).

Phagocytic cells of the immune system must detect and clear the multitude of cells that die as a result of normal homeostasis, development, stress, or various pathologic processes. This process, termed efferocytosis, is critical for the prevention of autoimmune and inflammatory disorders. Efferocytosis must be 'immunologically silent', otherwise a highly detrimental inflammatory or immunological response may occur. The details of these processes are not fully known, but fragmentary information is forthcoming. It is known that LC3-associated phagocytosis (LAP) is triggered when an extracellular particle, such as a dead cell, engages a macrophage receptor during phagocytosis. The receptor-ligand interaction induces the translocation of autophagy machinery and LC3 to the phagosome, termed the LAPosome (Green et al. 2016). The pathways that coordinate ingestion of the dead cells and direct or impede the subsequent immune response are an area of growing interest, with obvious implications for therapy of autoimmune diseases such as systemic lupus erythematosis that involve accumulations of apoptotic cells and cell fragments.

See reviews by Deretic and Levine for more detailed information regarding interactions between autophagy, immunity and inflammation (Deretic et al. 2013; Deretic 2005, 2006; Levine and Deretic 2007; Levine et al. 2011).

1.9.4 Microbiome and Immunity

Recent immunological research has evolved from a focus on lymphoid tissues to an understanding of local tissue environments as a fundamental determinant of immune responses. Local tissue conditions often influence local immune responses, and may modify local immune cell populations to meet the local tissue conditions and requirements. In addition to these conditions and requirements of local host tissues, the presence of microbial populations in and on the host tissues are also known to influence immune responses.

Each microbiome habitat of the host body is inhabited by different phyla of organisms, with niche-specific variation in the abundance and distribution of various species in different sites (Human Microbiome Project C 2012). The mammalian gut microbiome is a collection of bacteria, archaea, viruses, fungi, protozoa and metazoan parasites that reside in the lumen of the intestine (Howitt et al. 2016). The population of microbes in the gut increases from the stomach to the colon (Eckburg et al. 2005; Donaldson et al. 2016), with the human colon being home to an estimated 10^{13} microbial cells (Sender et al. 2016). The gut-associated microbiome performs vital functions such as preventing colonization by pathogens (Bohnhoff et al. 1954; van der Waaij et al. 1971), stimulation of immune system development and function (Bouskra et al. 2008; Mazmanian et al. 2008), and production of micronutrients that are utilized by the host (Donohoe et al. 2012). Deviation from normal microbiome composition, known as dysbiosis, has been associated with human diseases such as diabetes (Qin et al. 2012), heart disease (Koeth et al. 2013), arthritis (Scher et al. 2013), and malnutrition (Smith et al. 2013). Restructuring of the intestinal microbiome occurs rapidly following exposure to some xenobiotics. For example, exposure of adult zebrafish to triclosan-laden feed for only 4–7 days resulted in altered composition and ecological dynamics of microbial communities in the gut (Gaulke et al. 2016). There is evidence that restructuring of the gut microbiome may occur following ingestion of common food additives such as artificial sweeteners (Suez et al. 2014).

The bacterial population of the gut makes a major contribution to the homeostatic well-being of the host. Undigested complex hydrocarbons are abundant substrates for bacterial fermentation in the colon, resulting in the formation of short-chain fatty acids (SCFAs), which are energy sources for intestinal epithelial cells as well as bacterias. In addition, SCFAs have numerous regulatory functions on host physiology and immune functioning. SCFAs are inhibitors of histone deacetylases (HDACs) and serve as ligands for G protein-coupled receptors, thus act as signaling molecules that influence the expansion and function of hematopoietic and non-hematopoietic cell lines. Inhibition of HDACs by SCFAs constitutes an epigenetic regulator that promotes a tolerogenic, anti-inflammatory immune cell phenotype (Rooks and Garrett 2016). SCFA-induced HDAC inhibition is also a crucial regulator of NF-κB activity which commonly results in pro-inflammatory immune responses (Usami et al. 2008). SCFAs directly influence T cells, particularly Tregs, through HDAC inhibition (Tao et al. 2007). SCFAs fortify the barrier

function of intestinal epithelial cells by increasing the transcription of mucin genes in goblet cells (Willemsen et al. 2003; Gaudier et al. 2004) and strengthening the tight junctions between intestinal epithelial cells (Fukuda et al. 2011).

Members of the gut microbiota generate metabolites that can bind the aryl hydrocarbon receptor (AHR), a ligand-inducible transcription factor expressed by immune cells, epithelial cells and some neoplastic cells AHR was initially recognized due to its induction and interaction with certain classes of carcinogenic xenobiotics, but more recent studies have focused on the involvement of AHR in immune system homeostasis. Deficiencies in AHR activity cause alterations in the gut microbial population as well as reductions in the epithelial barrier function and lymphoid cell populations of the intestine (Rooks and Garrett 2016).

Polyamines such as putrescine, spermidine and spermine are polycationic molecules found in most living cells. Intestinal epithelial cells rely on the high level of polyamines produced by luminal microbes and host intestinal cells to maintain rapid turnover and high proliferation rate (Rooks and Garrett 2016). In addition to their involvement in epithelial cell dynamics, intestinal polyamines stimulate the production of intercellular junction proteins such as occludin, zonula occludens 1, and E-cadherin that are critical in maintaining the barrier function of the intestinal epithelial cells (Chen et al. 2007; Liu et al. 2009). Polyamines further contribute to immunity by inducing the production of mucus and secretory IgA in the intestine (Dufour et al. 1988; Buts et al. 1993). Polyamines also modulate systemic and mucosal adaptive immunity, apparently contributing to maturation of intraepithelial $CD8^+$ T cells and $CD4^+$ T cells in the intestinal lamina propria (Miller-Fleming et al. 2015).

The gut microbiota are separated from the host by a thin layer of epithelial cells that express germline-encoded pattern recognition receptors which detect microorganism-associated molecular patterns (MAMPs) of bacterial, fungal or viral origin and subsequently orchestrate mucosal immunity. The detected MAMPs most commonly are lipopolysaccharide, flagellin, peptidoglycan, formyl peptides or unique nucleic acid structures. Signaling through the innate immune system cascades into the adaptive immune system. Continual input from the innate immune system, which is initiated by exposure to gut microbes, is essential for maintenance of the mucosal and system adaptive immune system.

Polysaccharide A (PSA) has been extensively studied for its role in maintenance of adaptive immunity. PSA is produced and exported by *Bacteroides fragilis*, a commensal organism located in the superficial mucus layer of the colonic mucosa. PSA interacts with TLR2 on DCs (Wang et al. 2006), and is sampled, processed and presented to T cells by DCs (Mazmanian et al. 2005; Dasgupta et al. 2014). PSA can suppress intestinal inflammation by driving IL-10 production by $CD4^+$ T cells as well as increasing the population and activity of Tregs (Round et al. 2011; Mazmanian et al. 2008). In addition to these effects on mucosal adaptive immunity, PSA is also known to modulate systemic immunologic activities such as neuroinflammation (Wang et al. 2014b).

See (Rooks and Garrett 2016) for review of intestinal microbiome and immunity.

The skin is highly exposed to the environment and acts as the first line of immunological defense, but is also inhabited by many bacteria, fungi and viruses. In order to overcome the host defense systems, the cutaneouis microbes must overcome innate immune system components such as antimicrobial peptides (AMPs), proteases and reactive oxygen species. The microbes and host normally exist in a commensal relationship, with microbes assuming the role of pathogen only in certain situations.

The skin is composed primarily of epidermis and dermis. The epidermis consists of interfollicular epidermis and appendages such as follicles and associated structures. Keratinocytes of the epidermis form a physical and immunological barrier (Roberts and Horsley 2014). The dermis contains numerous immunologically active cells, including macrophages, dendritic cells, mast cells, innate lymphoid cells (ILCs, including group 2 ILCs and γδ T cells), and resident $CD4^+$ and $CD8^+$ T cells (Pasparakis et al. 2014; Tong et al. 2015). The skin initially becomes populated by microbes during childbirth, and even babies delivered by caesarean section acquire a typical population of skin microbes early in life (Mueller et al. 2015). The relative immaturity of the neonatal immune system promotes a tolerogenic immune response to the cutaneous microbes, probably involving Tregs (PrabhuDas et al. 2011; Belkaid and Tamoutounour 2016). During puberty the childhood population of cutaneous microbes shifts to include a dominant population of lipophilic bacteria (Oh et al. 2012), most likely a result of hormone-associated alterations in the activity of sebaceous glands. The skin of adult humans typically is home to more than 10^{10} bacteria (Belkaid and Segre 2014), but this number varies depending on hygiene practices (Bouslimani et al. 2015). Different areas of skin vary in microbial populations due to differences in local pH, temperature, moisture content and sebum content (Grice et al. 2009; Costello et al. 2009).

In the intestine the presence of microbes is necessary for establishment of GALT, but microbial populations are not necessary for establishment of cutaneous immune cell populations (Belkaid and Hand 2014; Naik et al. 2012). However, cutaneous microbial populations govern the expression of AMPs such as cathelicidins and β-defensins (Gallo and Hooper 2012). Commensal organisms on the skin compete with potential pathogens for space and/or defined metabolites, resulting in 'colonization resistance' to pathogens (Buffie and Pamer 2013; Belkaid and Hand 2014). Commensal microbes may also produce antimicrobial peptides that inhibit the growth of pathogenic microbes. For example, the dominant skin commensal *Staphylococcus epidermidis* produces several antimicrobial proteins and proteases that limit the proliferation of pathogenic *Staphylococcus aureus* (Iwase et al. 2010). Cutaneous microbes fine-tune the innate milieu, in particular promoting the production of IL-1, which in turn influences skin-homing T cells to produce the IL-17 and IFN-γ that are critical to defense against pathogens such as *Candica albicans* (Naik et al. 2012; Naik et al. 2015). Some cutaneoous microbes take an active role in suppression of inflammation. For example, binding of lipoteichoic acid from *Staphylococcus epidermidis* to TLR2 inhibits inflammatory responses, limits tissue damage and promotes wound healing (Lai et al. 2009).

See (Belkaid and Tamoutounour 2016) and (Pasparakis et al. 2014) for reviews of cutaneous immunity.

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Chapter 2
Cells of the Immune System

George A. Parker

Abstract A broad array of cells with immunological function are subcategorized as innate immune cells, adaptive immune cells, or cells of non-immune organs that have secondary immunological functions. These subcategories are convenient for purposes of discussion, but extensive observations indicate there are many overlaps between cells of the various subcategories. Macrophages, in particular, occupy a functional space that is shared between the innate and adaptive immune systems. Macrophages, neutrophils, eosinophils, basophils, mast cells, and NK cells of the innate immune system are known to influence the immunological activities of the various lymphocyte populations of the adaptive immune system. Conversely, cells of the adaptive immune system commonly enhance, repress, guide or direct the activities of cells of the innate immune system. A general knowledge of the roles played by the various immune system cells is critical to understanding immunological responses, particularly those immunological responses that are commonly encountered in non-clinical toxicology studies. Histopathological evaluation of tissue specimens is a major component of the analysis of non-clinical toxicology studies, and relatively subtle shifts in cell populations in various tissues can have major implications with regard to xenobiotic-mediated effects. The aim of this chapter is to present an overview of immune system cells and their functions, with a focus on cells and actions that are commonly important in the analysis of non-clinical toxicology studies.

Keywords Neutrophil • Monocyte • Macrophage • Eosinophil • Basophil • Myeloid suppressor cell • Mast cell • NK cell • NKT cell • MAIT cell • Dendritic cell • T lymphocyte • B lymphocyte • Stromal cell

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2.1 Introduction

In adult mammals the thymus and bone marrow are considered primary lymphoid organs. The secondary lymphoid organs (SLOs) include lymph nodes, spleen, and various components of mucosa-associated lymphoid tissue (MALT). Structures that are classically considered as MALT include Peyer's patches and diffuse intraepithelial lymphocyte populations (IEL) of the small intestine, nasopharynx-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT) of the lung, genital-associated lymphoid tissue (GENALT), and large intestinal lymphoid tissue. Less-known mucosa-associated lymphoid structures include cryptopatches (CP), isolated lymphoid follicles (ILFs), and lymphocyte-filled villi of the small intestine. Secondary lymphoid organs may be inducible or constitutively expressed, while tertiary lymphoid follicles (e.g., those seen in follicular conjunctivitis) are invariably induced by inflammation.

Within the multiple organs of the immune system and non-immune organs that have some level of immune functioning is a broad spectrum of cells that have primary or secondary roles in immune function. The goal of this chapter is to present basic immunobiological features of those cells and, to the extent possible in this limited space, present an overview of functional interactions between various cells of the immune system.

2.2 Primary Immune System Cells

Immune system cells formed during embryological development of mammals may originate from the yolk sac, aortic gonadal mesonephros region, or liver, but primary immune system cells of the adult mammal originate primarily from the bone marrow. Sequential steps in cell development in the marrow are well known (Fig. 2.1), and are incorporated into long-established procedures for cytological analysis of bone marrow. Recent years have seen major advances in knowledge regarding peripheral development of immune system cells, as well as interactions between bone marrow stromal elements and developing immune system cells. Both the bone marrow-related (central) and peripheral development of immune system cells may constitute the direct basis for disease processes, some of which may be amenable to pharmacological intervention. These processes may also present targets for pharmacological intervention in the immunological component of additional disease processes.

2.2.1 Cells of the Innate Immune System

Cells of the innate immune system provide an early response to pathogen invasion that does not involve the delays associated with the cell activation and replication seen in the adaptive immune system. Until recently the central dogma maintained

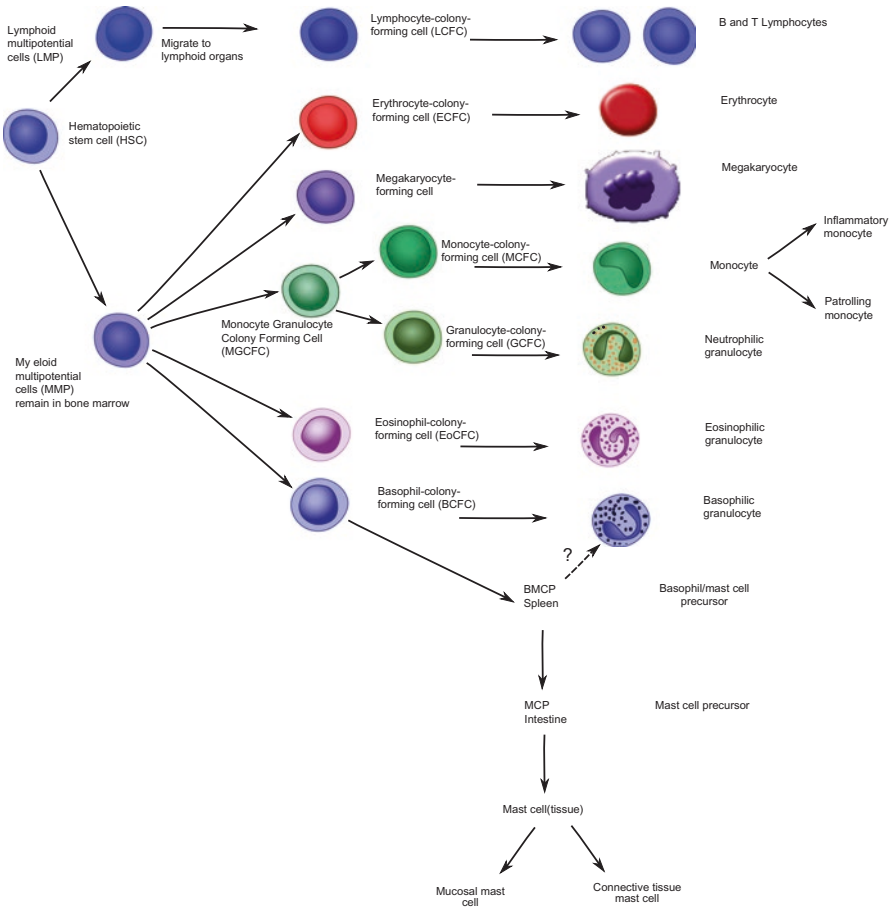

Fig. 2.1 This schematic gives an overview of immune cell generation in the bone marrow and secondary lymphoid organs

that only the adaptive immune system employed immunological memory, but this dogma has been challenged by observations that plants and invertebrates, which lack adaptive immune responses, can develop resistance to reinfection (Netea et al. 2016). After infection or vaccination of mammals, innate immune cells such as monocytes, macrophages and NK cells display long-term changes that increase their responsiveness upon second exposure to pathogens. The changes in innate immune cells involve epigenetic changes such as altered transcription programs and physiology, as opposed to the permanent genetic changes seen in memory cells of the adaptive immune system. The epigenetic changes include histone modification with chromatin reconfiguration, DNA methylation, and modulation of microRNA (miRNA) and long non-coding RNA (lncRNA) expression. This process, known as 'trained immunity' or 'innate immunological memory', provides numerous opportunities for development of novel vaccines, new therapeutic strategies for treatment

of immunodeficiencies, and modulation of inflammation in autoimmune diseases (Netea et al. 2015; Netea et al. 2016).

In addition to directly combating microbial pathogens, innate immune cells are involved in the induction of adaptive immune responses by internalization of pathogens and dying cells, with subsequent processing of peptides which are expressed in MHC context to activate αβT cells. Innate immune cells are also involved in direct recognition of danger and inflammatory signals with subsequent downstream signals that lead to functional polarization of B cell and αβ T cell responses (Banchereau and Steinman 1998).

This chapter is concerned primarily with cells of the immune system, but it should be recognized that the innate immune system has both cellular and humoral arms (Bottazzi et al. 2010). The humoral arm includes pattern recognition molecules such as colectins, ficolins and pentraxins, which have an antibody-like function in interacting with conserved microbial molecules. These humoral molecules are discussed in greater detail below under Neutrophils.

2.2.1.1 Monocytes and Macrophages

Circulating monocytes are bone marrow-derived phagocytes that participate in numerous immune responses, including in innate responses to infectious and parasitic agents (Barbalat et al. 2009; Gupta et al. 2001; Serbina et al. 2009a; Serbina et al. 2009b; Serbina et al. 2008; Stroher et al. 2001). Recent evidence suggests monocytes are not a homogeneous population and, similar to the situation with B and T lymphocytes, different functions are associated with distinct subpopulations of monocytes. Monocytes of mice have been subdivided into inflammatory ($Gr1^+$) and patrolling ($Gr1^-$) subsets. Similar monocyte subsets have been identified in humans (Cros et al. 2010). Inflammatory (or classical) monocytes of mice respond to bacterial or protozoan infections by producing inflammatory mediators such as TNF-α, reactive oxygen species, and nitric oxide, and to viral infection by producing interferons (Barbalat et al. 2009; Serbina et al. 2008). Patrolling (or nonclassical) monocytes of mice patrol the vasculature, where they scavenge microparticles and remove cellular debris (Auffray et al. 2007; Carlin et al. 2013; Cros et al. 2010). Patrolling monocytes mature into macrophages after emigration into tissues, and may be associated with tissue repair following injury (Auffray et al. 2007; Nahrendorf et al. 2007). Patrolling monocytes retain their tendency to patrol vascular endothelium after adoptive transfer, suggesting this is an inherent rather than a host-influenced function of this monocyte sub-population (Cros et al. 2010). Inflammatory versus patrolling monocytes have markedly different functions in tumor biology. Inflammatory monocytes are recruited to tumor sites, where they mature into macrophages that promote tumor growth and metastasis (Movahedi et al. 2010; Qian et al. 2011). Patrolling monocytes are enriched in the microvasculature of the lung and have been shown to reduce tumor metastasis in multiple mouse metastatic tumor models, thus the immunosurveillance function of patrolling monocytes may be a target for cancer immunotherapy (Hanna et al. 2015).

Macrophages are widely distributed throughout the mammalian body and have myriad functions, including phagocytic clearance of pathogens and cell debris, production of cytokines, chemokines and eicosanoids, and multiple modulations of other lymphoid cells. Macrophages are characterized by their extended half-life (Gordon 2013) and potential for self-renewal (Sieweke and Allen 2013). Macrophages have a well-known ability to adapt to different tissue locations and assume characteristics that are appropriate to their function within that tissue. Within a given organ, macrophages may also adapt different functional characteristics depending upon their sub-location within the organ. For example, macrophages in different regions of lymphoid tissues may be dedicated to either antigen presentation or phagocytic clearance of particulate material, including cell debris (den Haan and Martinez-Pomares 2013).

Macrophages are primary effector cells of both the innate and adaptive immune systems, and have highly specialized functions that are served by specific adaptations of the macrophages as well as precise locations within the tissues. Macrophages often are strategically located to impact on both innate and adaptive immunity, and are major producers of cytokines (Wynn et al. 2013). In the bone marrow, where most mammalian hematopoiesis occurs, macrophages are involved in the mobilization of hematopoietic stem cells (HSCs) from the endosteal hematopoietic niche via modulation of CXCL12 production by $Nestin^{+}$ mesenchymal stem cells (MSC) (Winkler et al. 2010; Chow et al. 2011; Ehninger and Trumpp 2011) and preservation of primitive HSCs via production of prostaglandin PGE_2 (Ludin et al. 2012). In the thymus, macrophages are responsible for removing the cellular debris that results from positive and negative selective processes and subsequent apoptosis of more than 90% of newly formed T cells (Surh and Sprent 1994). The phagocytosis of apoptotic fragments is dependent on expression of phosphatidylserine on the surface of apoptotic cells (Ravichandran 2011), which results from alterations in membrane phospholipid transferase ('flippase') enzymes that occur during the apoptotic process (Segawa et al. 2014).

Macrophages in different regions of lymphoid tissues may be dedicated to either antigen presentation or phagocytic clearance of particulate material, including cell debris (den Haan and Martinez-Pomares 2013). This characteristic is particularly noteworthy in the spleen, where multiple macrophage populations have different functions. Macrophages located near the marginal sinuses, the site of blood entry into the spleen, are focused on removal of apoptotic cells (Miyake et al. 2007), while macrophages in the red pulp focus on scavenging effete erythrocytes and recycling their iron content (Ganz 2012). The splenic red pulp macrophages and Kupffer cells of the liver have similar high-level exposure to blood and are positioned to remove aged erythrocytes that have lost the CD47 'don't eat me' signal (Amit et al. 2015). Engulfed erythrocyte-origin heme induces expression of Spi-C, a transcription factor that maintains the heme-metabolizing processes of the splenic red-pulp macrophages and hepatic Kupffer cells (Haldar et al. 2014).

In secondary lymphoid tissues such as the spleen and lymph nodes, sites of incoming antigen exposure have significant populations of $CD169^{+}$ macrophages (den Haan and Martinez-Pomares, 2013). $CD169^{high}$ macrophages are located in the

subcapsular sinuses of lymph nodes and marginal zones of the spleen ('marginal metallophilic macrophages'), where their proximity to B cell follicles promotes immune recognition, while CD169^{low} macrophages are located in the red pulp and outer marginal zone of the spleen and medulla of lymph nodes, where they are involved in clearance of particles and molecules. The phagocytic function of CD169^{low} macrophages in the spleen is of particular interest to toxicologists and toxicologic pathologists because of the role these macrophages have in clearing effete erythrocytes from the circulation. When test articles with oxidizing properties bind to erythrocytes, the deformability of the erythrocytes may be reduced. Splenic macrophages rely to some extent on this characteristic of erythrocytes to determine whether individual erythrocytes should be removed from the circulation. Thus test article-related oxidation of erythrocyte membranes may result in hematologic evidence of anemia, and possible additional adverse effects on the spleen if the oxidative test article remains bound to erythrocytes and there is a high cumulative dose of test article in the spleen.

In addition to their numerous immunological functions, macrophages have long been known for their ability to rid the body of pathogens, tissue debris and particulate matter through the process of phagocytosis. More recent information indicates that macrophages have the ability to adapt to various stimuli and assume diverse phenotypes that support a broad array of functions such as tissue development, systemic metabolism, tissue homeostasis, and cold adaptation. Macrophages can switch between phenotypes, depending on the needs of the local tissue environment. Macrophages in all locations were historically thought to have a shared origin from monocytes that originate in the bone marrow, but it is now known that a second population of macrophages originates during fetal life from three overlapping waves of precursor cells and persist for a prolonged period as tissue-resident macrophages that do not require replenishment from the bone marrow.

In contrast to hematopoietic-origin macrophages, tissue-resident macrophage populations are largely established prenatally and persist throughout life without additional hematopoietic input (Perdiguero and Geissmann 2015; Schulz et al. 2012; Bain et al. 2014; Varol et al. 2007; Molawi et al. 2014; Yona et al. 2013). For example, microglial cells of the brain, the archetypal tissue-resident macrophages, arise from yolk sac-derived erythromyeloid precursors that enter the neuroepithelium early in embryogenesis and subsequently undergo local proliferation (Ginhoux et al. 2010; Kierdorf et al. 2013). Most of the long-lived tissue-resident macrophages are derived from these embryonic precursor cells rather than hematopoiesis in adults (Schulz et al. 2012; Hashimoto et al. 2013; Yona et al. 2013). By contrast, tissues exposed to large populations of microbes, such as the skin and intestinal tract, are populated primarily by monocyte-derived macrophages that originate from bone marrow hematopoiesis (Malissen et al. 2014; Zigmond and Jung 2013).

Adaptation of macrophages to assume certain accessory functions is part of a general pattern exhibited by specialized cells and tissues. As cells of metazoan organisms mature and specialize during phylogeny and ontogeny, the specialized cells tend to abdicate some of their functions to various types of accessory cells.

Tissue-resident macrophages serve in this population of accessory cells, which can assume a myriad of functions as determined by tissue identity signals as well as functional demands imposed by specific conditions in the tissues. The tissue-resident macrophage population may have characteristics that are largely determined by tissue identity signals, with relatively minor alterations due to tissue condition signals. Tissue-specific adaptations of macrophage populations may allow them to provide niche-specific development or survival factors for other cell populations, e.g., the development of hematopoietic cells in the bone marrow or mature lymphoid cells in secondary lymphoid organs. In times of crisis, the tissue-resident macrophage population is aided by an influx of bone marrow-derived macrophages that are likely to have general characteristics determined largely by local tissue conditions, e.g., inflammation, apoptosis or necrosis, with less influence by the tissue-related signals that govern the tissue-resident macrophage populations.

All immune system cells have identical genomes, yet the cells exhibit major phenotypic and functional differences. Variations in chromatin structure have a major impact on the final characteristics of the cells (Capucha et al. 2015; Heinz et al. 2010; Ostuni and Natoli 2013; Winter and Amit 2014; Garber et al. 2012). The fundamental unit of chromatin is the nucleosome, which consists of approximately 147 DNA bases wrapped around a histone octet consisting of two molecules each of histones H2A, H2B, H3 and H4 (Felsenfeld and Groudine 2003). The DNA strand wraps around sequential histone octets, which are packaged into solenoid structures and higher forms of DNA structure (Butler 1984). Gene expression is directly regulated by methylation, acetylation, phosphorylation, ubiquitination and sumoylation of the histone molecules (Kouzarides 2007; Amit et al. 2015). Modification of DNA by these processes is known as ‘epigenomics’, which is evolving as an important modulator of immune cell structure and function. The tissue-specific differentiation of resident macrophages is determined by epigenetic mechanisms such as DNA methylation, histone modification and variation in chromatin structure (Amit et al. 2015). These epigenetic factors are largely controlled by lineage- and tissue-specific transcription factors that control development of myeloid cells in combination with tissue environment factors. Elucidation of epigenomic modifications has promise as a definitive basis for classifying various immune cell populations, and may provide opportunities for pharmacological influence on disease processes (Amit et al. 2015).

Macrophages and dendritic cells are presented herein as separate cell populations, as is current custom, but there is evidence the appellations of ‘macrophage’ and ‘dendritic cell’ may merely reflect different functions of the same cell population. (This comment does not apply to tissue-resident macrophages and follicular dendritic cells, which are separate from the hematopoietic-origin cell populations addressed here.) See (Hume 2008) for further details on the history of macrophage terminology and ‘the dendritic cell myth’.

Pathology terminology related to macrophages may be confusing, particularly to non-pathologists, but it allows for more efficient communication once the terminology becomes familiar. Macrophages that are engorged with phagocytosed or otherwise accumulated material are typically termed ‘histiocytes’ in most affected

tissues. Sinus histiocytosis refers to accumulations of engorged macrophages in the subcapsular, paracortical and medullary sinuses of lymph nodes, while alveolar histiocytosis refers to engorged macrophages within pulmonary alveoli. Fusion of macrophages or histiocytes results in the formation of multinucleated giant cells, which may be of the foreign body type (nuclei in a central cluster) or Langerhans type (nuclei arranged around the periphery of the giant cell). Macrophages and histiocytes are sometimes called epithelioid cells, which refers to the histologic similarity between active macrophages and epithelial cells. Tissue-adapted macrophages commonly have tissue-specific names, e.g., microglial cells of the brain and Kupffer cells of the liver. Over the years the terminology has been further complicated by application of additional specific names to tissue-associated macrophages that have histologic features which support sub-classification, e.g., 'gitter cell' to indicate engorged microglial cells of the brain.

Macrophages are a component of most inflammatory reactions, and may be the primary cellular component in inflammatory lesions classified as granulomatous. The term 'granulomatous' is derived from the histologic appearance of tuberculosis-associated granulomas, which typically contained numerous macrophages and multinucleated giant cells. The term is now used to denote virtually any inflammatory lesion in which macrophages are a prominent histologic component, and does not necessarily imply presence of classical granulomas.

Macrophage-related histologic changes are commonly encountered in toxicologic pathology. Macrophages are a constituent of many inflammatory lesions, and may be the primary cellular feature of granulomatous inflammation. Pulmonary macrophages laden with anthracosilicotic pigment associated with polluted air are commonly seen in the lungs of nonhuman primates, and must be distinguished from the pigment-laden macrophages that accompany *Pneumonyssus* spp. lung parasitism. Sinus histiocytosis is a very common background finding in the lymph nodes of aged rodents, and minor accumulations of alveolar macrophages are seen in the lungs of most species. Assignment of toxicologic significance to the presence of alveolar macrophages can be problematic, as the decision is based largely on a subjective determination that the alveolar macrophage population is increased in test article-treated animals. The potential pathologic significance of any alveolar macrophage accumulation is accentuated by the well-known adverse effects of macrophage engagement with asbestos, which constituted a major toxicologic disaster that seriously injured or precipitated the death of numerous individuals.

Not all forms of macrophage engorgement result from an increased rate of phagocytosis of extraneous material. As part of normal homeostasis, macrophages are involved in the degradation of numerous cellular products. Genetically determined or otherwise imposed defects in macrophage degradative function can result in accumulation of substrate molecules within macrophages, and result in microscopically visible macrophage engorgement. These changes are particularly noteworthy in genetically defined metabolic defects in the degradation of various CNS molecules, resulting in the engorged macrophages seen with globoid cell leukodystrophy ('Krabbe's disease', galactosylceramidase deficiency), etc.

2.2.1.2 Neutrophils

"Neutrophil" was the term used by Paul Ehrlich in 1880 to describe leukocytes with "polymorphous nuclei" and a tendency to retain neutral dyes (Amulic et al. 2012). Neutrophils are the most abundant circulating leukocytes in humans and some animal species, but are out-numbered by lymphocytes in other animal species. Regardless of their numerical predominance, neutrophils have a central role in innate immunity, and there is evolving evidence that neutrophils have extensive interactions with the adaptive immune system. Human neutrophils typically have a half-life of only 6–8 h in circulation, thus are among the shortest-lived cells in the body. However, as presented below, neutrophils continue to influence inflammatory reactions after the demise of the neutrophils at the site of inflammation.

Neutrophils emigrate from blood vessels to sites of inflammation via a well-characterized stepwise process that includes low-affinity selectin-mediated rolling, high-affinity integrin-mediated binding, and cellular egress and migration into the perivascular tissue. During these migratory steps the neutrophil is undergoing a series of internal changes that serve to 'activate' the neutrophil and make it into an effective microbe-killing weapon. See (Muller 2013) and Chap. 7 for a review of leukocyte emigration to sites of inflammation.

The antimicrobial proteins that are produced and transported by neutrophils are too dangerous to exist freely in the cytoplasm, thus are sequestered into three types of cytoplasmic granules known as azurophilic (or primary), specific (or secondary), and gelatinase (or tertiary) granules. Secretory vesicles may constitute a fourth type of protein containment compartment within neutrophils. The first three types of granules form by budding from the Golgi apparatus, but the secretory vesicles are formed via endocytosis (Borregaard et al. 2007). Primary granules are characterized by presence of myeloperoxidase. Secondary granules do not contain myeloperoxidase, and are characterized by presence of lactoferrin. Tertiary granules are also myeloperoxidase-negative, and characterized by presence of gelatinase. The granules and secretory vesicles are formed in the order named, with primary granules forming first and secretory vesicles forming last as the neutrophilic precursors mature in the bone marrow. This pattern is reversed during degranulation or mobilization of granules, with secretory vesicles first and primary granules the last to fuse with the plasma or lysosomal membrane (Amulic et al. 2012). The membrane of specific granules contains flavocytochrome B558, a component of the NADPH oxidase machinery, therefore, the final fusion of specific granules with the plasma or lysosomal membrane allows development of the microbicidal oxidase burst.

Neutrophils produce a myriad of antimicrobial molecules which can be generally categorized as (1) cationic peptides and proteins that bind to membranes, (2) enzymes, and (3) molecules that deprive bacteria of essential nutrients (Amulic et al. 2012). The cationic membrane-binding peptides include defensins and cathelicidins. The enzyme group includes lysozyme, which directly destroys bacterial cell walls, and a number of serine proteases. The third group includes lactoferrin, which binds to iron, and calprotectin, which sequesters zinc (Corbin et al. 2008; Weinberg 1975).

Neutrophils play a critical role in host defense via their role as 'professional phagocytes, which involves phagocytosis of particles larger than 0.5 μm diameter. Phagocytosis is triggered by neutrophil receptor recognition of polysaccharides on the surface of yeast cells, or binding of opsonized microorganisms to Fcγ and/or complement receptors. Microbial pathogens are internalized into cytoplasmic phagosomes, which subsequently fuse with neutrophil granules to form phagolysosomes.

Microbicidal killing by neutrophils involves an oxidative burst that results in formation of the reactive oxygen species (ROS) that are the penultimate killing tools. The NADPH oxidase complex that initially forms on the phagosomal and plasma membranes reduces molecular oxygen to superoxide. Superoxide can dismutate to hydrogen peroxide, or can react with nitric oxide to form peroxynitrite, either of which is microbicidal. Myeloperoxidase can react with hydrogen peroxide to form various reactive molecules, including hypochlorous acid. Hypochlorous acid is even more reactive than superoxide, and highly antimicrobial, but the chloramines that result from hypochlorous acid reaction with amine groups are the most lethal antimicrobial molecules in phagosomes (Winterbourn et al. 2006). As would be expected for such a critically important defensive system, there is substantial overlap and redundancy in the microbicidal killing systems.

As they respond to infection or injury, neutrophils and cells of the monocyte/macrophage lineage coordinate their activities, leading to alternating waves of recruitment of the two cell lineages. This immunologic process has led to the histopathological term 'chronic-active' inflammation, which implies chronicity associated with the macrophage population and active status associated with the neutrophil population. Macrophages and patrolling monocytes are among the earliest detectors of PAMPs and DAMPs (Cailhier et al. 2005), and downstream signaling from these cells results in neutrophilic infiltration into the area. The arrival of neutrophils in the area of inflammation leads to the recruitment of an additional population of monocytes via production of monocyte chemoattractants such as CCL2 (monocyte chemoattractant protein-1, MCP-1), CCL3 (macrophage inflammatory protein-1, MIP-1α), CCL20 (MIP-3α), and CCL19 (MIP-3β) (Scapini et al. 2001). In addition to recruiting monocytes and macrophages, neutrophils enhance the microbicidal activity of those cells (Soehnlein et al. 2008).

The interactions between neutrophils and monocytes/macrophages continue as the inflammatory reaction wanes. The monocytes recruited by neutrophils subsequently differentiate into macrophages, which repress further neutrophil chemotaxis and eventually remove debris from the site of inflammation. Neutrophil involvement in inflammatory reactions is somewhat plastic. Neutrophils that initially infiltrate into an area of inflammation produce pro-inflammatory lipid mediators such as prostaglandins and leukotrienes but, as the inflammatory reaction matures, neutrophils undergo a 'lipid class switch' to produce anti-inflammatory and pro-resolving mediators such as lipoxins, resolvins and protectins (Serhan 2007). In addition, the microbial pathogens that initiated the inflammatory reaction also participate in resolution of the inflammatory reaction by producing pro-resolving mediators (Arita et al. 2005; Vance et al. 2004).

Innate immune cells such as neutrophils quickly infiltrate into areas of inflammation from the circulating blood, but cells of the adaptive immune system require activation before they become functional. This constitutes a delay in the involvement of adaptive immune cells such as lymphocytes in sites of inflammation. These basic features of immunobiology contributed to traditional histopathology terms such as 'acute' inflammation for inflammatory cell infiltrates consisting primarily of neutrophils, and 'subacute' inflammation for infiltrates in which lymphocytes predominate. This general system is evolutionarily conserved, as the slime mold *Dictyostelium discoideum* uses a similar strategy to organize the head-to-tail orientation when the single-celled organisms stream together to form a multicellular aggregate (Kriebel et al. 2003). This system is another example of exteriorized cellular components functioning in immunologic signaling (Colombo et al. 2014).

Neutrophils have long been viewed as short-lived effector cells of the innate immune system, characterized by their phagocytic ability, release of lytic enzymes from their cytoplasmic granules, and production of reactive oxygen species with antimicrobial properties (Borregaard 2010; Nathan 2006). More recent evidence suggests neutrophils have more extensive involvement in immunity (Mantovani et al. 2011). Neutrophils express inflammatory mediators such as complement components, Fc receptors, chemokines and cytokines (Cassatella 1999). Neutrophils may also produce anti-inflammatory molecules that help resolve inflammation. As is seen with many other immune cell populations, neutrophils can be polarized toward distinct phenotypes in response to environmental signals (Fridlender et al. 2009). Some of these signaling processes represent potential pharmaceutical targets. For example, TGF-β in a tumor microenvironment induces a pro-tumor population of tumor-associated neutrophils (TANs) that serve to promote tumor growth and inhibit adaptive immune responses to the tumor, while TGF-β blockade results in a subpopulation of TANs that are hypersegmentated, more toxic to tumor cells, and express higher levels of proinflammatory cytokines

In addition to their key role as 'microphages' and microbe killers in innate immunity, neutrophils interact with various components of the adaptive immune system. T_H17 cells produce mediators such as IL-17, the chemokine CXCL8 (also known as IL-8), interferon-γ, tumor necrosis factor (TNF), and granulocyte-macrophage colony stimulating factor (GF-CSF), all of which serve to recruit, activate, and prolonge the survival of neutrophils at sites of inflammation (Cua and Tato 2010). T_H17 lymphocytes produce IL-17 and related cytokines that promote granulopoiesis and subsequent neutrophil proliferation and accumulation.

The AUG start codon, which initiates the reading frame of mRNA, codes for methionine in mammalian systems, but codes for formyl-methionine in prokaryotic systems. Formylated peptides produced by bacteria or mitochondria (see endosymbiont hypothesis) serve to activate the seven-transmembrane G protein-coupled receptor FPR1 on neutrophils and promote neutrophil chemotaxis to sites of inflammation (Zhang et al. 2010; McDonald et al. 2010). FPR1 thus serves as a pattern recognition receptor (PRR) in this component of the innate immune system. Neutrophils also express a number of Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and cytoplasmic sensors of nucleic acids (RIG-1 and MDA), and

nucleotide-binding oligomerization domain protein 1 (NOD1), all of which may serve to activate downstream events that trigger the innate immune functions of neutrophils (Tamassia et al. 2008; Clarke et al. 2010).

Neutrophils produce an abundance of cytokines, as reviewed in (Cassatella 1999, 1995) and summarized by (Mantovani et al. 2011). A number of neutrophil-origin cytokines are not produced upon demand, but instead are pre-formed and stored in intracellular pools for immediate release upon appropriate stimulation, thus helping to characterize neutrophils as part of the early-response team.

Neutrophils are a major source of cytokines that are essential for survival, maturation and differentiation of B cells. Included are B cell-activating factor (BAFF) and a closely related proliferation-inducing factor known as APRIL (Scapini et al. 2008; Huard et al. 2008). High levels of APRIL are produced by neutrophils in the synovial fluid of patients with rheumatoid arthritis, inflamed mucosa-associated lymphoid tissue (MALT), and B cell malignancies (Huard et al. 2008; Gabay et al. 2009; Roosnek et al. 2009). It appears that APRIL serves to sustain autoantibody production as well as promote the growth and progression of B cell malignancies. This is another facet of the common theme that connects pharmacological modulation of immune system function to treatment of both autoimmune disease and neoplasia.

In addition to maintaining the B cell component of the inflammatory reaction in patients with rheumatoid arthritis, neutrophils also produce a ligand (RANKL) for the receptor activator of NF-κB (RANK), which activates osteoclasts and results in bone resorption. This function of neutrophils may be involved in the well-known tendency toward bone resorption in association with inflammation.

Neutrophils contribute to the humoral arm of the innate immune system by releasing soluble pathogen recognition molecules (PRMs) that enhance phagocytosis, activate complement, and regulate inflammation. Neutrophils are a major reservoir of preformed pentraxin X3 (PTX3), which is stored in intracellular pools and released immediately upon proper stimulation (Jaillon et al. 2007). PTX3 and other neutrophil-origin molecules contribute to the microbicidal properties of neutrophil extracellular traps (NETs), as discussed below.

Contrary to conventional wisdom, not all of the neutrophils recruited to sites of inflammation are destined to die at the site. Neutrophils can capture antigens and then migrate from sites of inflammation to regional lymph nodes, similar to the pathway taken by dentritic cells (Abadie et al. 2005; Chtanova et al. 2008; Beauvillain et al. 2011). This neutrophil migration to regional lymph nodes is no surprise to histopathologists, who commonly observe a minor neutrophilic population in ‘reactive lymph nodes’ that drain areas of inflammation.

There is substantial cross-talk between neutrophils and other immune system cells, including macrophages, dendritic cells, NK cells, lymphocytes and mesenchymal stem cells (Borregaard 2010; Mantovani et al. 2011; Soehnlein and Lindbom 2010). Neutrophils modulate the activation status of NK cells, and are required for the homeostatic maintenance of NK cell populations. There is a reciprocal relationship between NK cells and neutrophils, as culture of neutrophils with NK cells or NK cell-derived soluble factors promotes neutrophil survival and func-

tion (Costantini and Cassatella 2011). In their interactions with the adaptive immune system, neutrophils positively support T_H1 responses in preference to T_H2 responses. Neutrophils directly prime antigen-specific T_H1 and T_H17 cells (Abi Abdallah et al. 2011), and neutrophil-influenced dendritic cells induce T cell polarization toward a T_H1 phenotype (Megiovanni et al. 2006; van Gisbergen et al. 2005). Activated neutrophils attract T_H1 and T_H17 cells to site of inflammation via release of specific chemoattractants (Pelletier et al. 2010). Conversely, activated T cells can recruit neutrophils. Neutrophils recruit and activate dendritic cells as well as secrete IL-12, which is important in T_H1 cell differentiation (Peters et al. 2008; Romani et al. 1997; Tateda et al. 2001). Neutrophils have multiple interactions with NK cells, sometimes involving three-way interactions between neutrophils, NK cells and dendritic cells (Costantini and Cassatella 2011). Neutrophils have some characteristics of antigen-presenting cells, including expression of MHC class II and costimulatory molecules under inflammatory conditions, and can present antigen to $CD4^+$ T cells in vitro (Fanger et al. 1997; Radsak et al. 2000; Culshaw et al. 2008).

Some communication between neutrophils and other immune cells does not involve direct cell-to-cell communication. Neutrophils leave a trail of extracellular fragments containing CXCL12 that guide $CD8^+$ T cells to sites of inflammation (Lim et al. 2015; Kiermaier and Sixt 2015). Instead of directly secreting the CXCL12 polypeptide, neutrophils deposit the chemokine wrapped in membrane fragments that are deposited upon collagen fibrils in the region of inflammation. The deposited membrane fragments gradually release CXCL12, creating a prolonged promigratory and chemoattractive millieu for T cells (Mantovani et al. 2011). Thus the presence of neutrophils in an acute inflammatory reaction sets the stage for the subsequent lymphocytic infiltration that characterizes subacute and chronic inflammatory lesions.

As presented above, immune functions of neutrophils are not limited physically to sites of inflammation or functionally to direct effects of neutrophils on pathogens. Even more interesting are recent indications the activities of neutrophils are not limited to the lifespan of the neutrophils. Histopathologists have long recognized deposits of basophilic fibrillar material associated with inflammatory lesions caused by bacteria and fungi. These deposits were generally dismissed as an expected release of nucleic acids and proteins from damaged tissues, but recent evidence indicates the deposits represent an organized response of neutrophils known as neutrophilic extracellular traps (NETs). NETs consist of a backbone of neutrophilic DNA that supports histones, antimicrobial peptides and proteins derived from azurophilic, secondary and tertiary neutrophilic granules (Hermosilla et al. 2014). The expanding list of proteins contained within NETs includes myeloperoxidase, pentraxin, lactoferrin, gelatinase, bacterial permeability-increasing protein (BPI), cathepsin G, peptidoglycan recognition proteins (PGRPs) and calprotectin. The concentration of microbicidal molecules within a matrix that physically traps bacteria is highly effective at killing invasive bacteria but causes minimal damage to surrounding tissues (Fuchs et al. 2007; Ermert et al. 2009; Abi Abdallah and Denkers 2012; Hahn et al. 2013). NETs are known to exist in a large number of mammalian species as well as fish and insects (Hermosilla et al. 2014). Other cells such as

eosinophils, mast cells, and macrophages produce antimicrobial structures that are similar to NETs. In addition to their function in microbial killing, NETs are also involved in reproductive disorders and autoimmune diseases such as systemic lupus erythematosus (SLE) (Alghamdi and Foster 2005; Logters et al. 2009). NET generation is reduced in neonates versus adults, which may predispose neonates to bacterial infections (Yost et al. 2009).

NETs are commonly produced by a controlled death pathway known as 'NETosis', which involves sequential dissembly of nuclear and granule membranes of neutrophils, mixing of nuclear and granule components, and extrusion of the NET complexes into the extracellular space. This process allows neutrophils to kill pathogens beyond the lifespan of the neutrophils (Brinkmann and Zychlinsky 2007). In addition to the classical pathway of NET formation, which requires death of the neutrophils, neutrophils may also release NETs without perishing in the process (Yousefi et al. 2009; Yipp et al. 2012).

The involvement of NETs in resistance to bacterial and fungal pathogens is well-established, but recent evidence suggests NETs are also involved in immune responses to viruses as well as protozoan and metazoan parasites (Hahn et al. 2013). In some diseases it appears that circulating NETs are involved in resistance to protozoan parasites such as *Plasmodium falciparum* (Baker et al. 2008), *Toxoplasma gondii* (Abi Abdallah and Denkers 2012), and *Leishmania* spp. (Guimaraes-Costa et al. 2009). NETs have also been shown to be involved in resistance to enteric protozoan pathogens such as *Eimeria* spp. (Behrendt et al. 2010) and metazoan parasites such as *Schistosoma japonicum* (Chuah et al. 2013). Pathogenic organisms can employ a wide array of strategies to avoid entrapment in NETs, including expression of DNAse enzymes that destroy the nucleic acid backbone of NETs (Buchanan et al. 2006; Beiter et al. 2006; Berends et al. 2010). The pathogens may also employ molecular mimicry (Khatua et al. 2012) or coat themselves with host molecules (Carlin et al. 2009) to avoid detection, or trigger the expression of immunomodulatory molecules such as IL-10, which dampens the NET-formation capability of neutrophils (Carlin et al. 2009). These pathogen-associated avoidance strategies are targets for antimicrobial therapy (Peterson et al. 2013). See (Hahn et al. 2013) for a review of NET versus pathogen interactions.

There is emerging evidence that neutrophils may have a role in cancer development and metastasis. Microscopic evidence of neutrophilic infiltration in neoplasms has long been viewed as a negative prognostic feature, based on the presumption that the neoplasm disrupted local homeostasis to the degree that an inflammatory reaction resulted. Elevated circulating neutrophil level in cancer patients is associated with increased risk for metastasis (Templeton et al. 2014). Recent evidence suggests neutrophil accumulation in distant organs occurs in advance of the arrival of metastatic tumor cells, and enhances the early steps in metastasis. In one mouse model, neutrophils localize in the lung and produce leukotrienes that facilitate tumor cell colonization (Wculek and Malanchi 2015). In another mouse model, neutrophils in the lung hinder antitumor T cell immunity, a process that involves cooperation between neutrophils, γδ T cells and IL-17 (Coffelt et al. 2015). It is known that growing neoplasms in peripheral tissues perturb the process of bone

marrow granulopoiesis to result in neutrophils switching from a tumor-protective to a less mature, disease-promoting phenotype (Sagiv et al. 2015). However, this emerging evidence of tumor-promoting effects of neutrophils is balanced by evidence of antimetastatic effects of neutrophils (Granot et al. 2011; Finisguerra et al. 2015). Additional information is required before this potentially promising accessory treatment for cancer can be implemented clinically.

Pharmaceutical modulation of neutrophil involvement in tumor metastasis, whether positive or negative, has obvious potential clinical benefit. There is strong evidence that molecules such as the high mobility group box 1 (HMGB1), interleukin 17 (IL-17), and granulocyte colony-stimulating factor (G-CSF) promote the generation of protumor neutrophil phenotypes, and interference with these molecules results in a lower level of lung metastasis in mouse models (Bald et al. 2014; Coffelt et al. 2015; Casbon et al. 2015). Inhibition of the arachidonate 5-lipoxygenase that transforms fatty acids into leukotrienes impairs the formation of lung metastases of a mouse cancer model (Wculek and Malanchi 2015). Neutrophils in the lung produce proteases that degrade thrombospondin 1, which is an anti-tumor effector molecule, thus in this context neutrophils are pro-tumor. Interfering with the production of neutrophil proteases abrogates the effect on thrombospondin 1 and has the end result of reducing lung metastasis (El Rayes et al. 2015). These approaches to reducing the metastatic rate are particularly attractive because many potentially useful anti-metastatic drugs have already been developed as anti-inflammatory drugs.

See (Amulic et al. 2012) and (Mantovani et al. 2011) for reviews of neutrophil function. See Sect. 2.2.1.6 below for discussion of myeloid suppressor cells and their involvement in cancer immunobiology.

2.2.1.3 Eosinophils, Basophils & Mast Cells

Eosinophils, basophils and mast cells are important effector cells in allergic inflammation, and have a critical role in surveillance of the gastrointestinal, respiratory and urogenital tracts (Scapini et al. 2013). Mast cells are tissue-resident sentinels, while eosinophils and basophils are recruited from the circulating blood. In contrast to neutrophils, which destroy phagocytized pathogens by delivering toxic molecules into phagosomes, eosinophils, basophils and mast cells are commonly involved in resistance to large pathogens that cannot be phagocytized by individual host defense cells. As presented in greater detail below, eosinophils produce molecules that are directly toxic to large pathogens, while basophils and mast cells release molecules that render the extracellular milieu hostile to the large pathogens. Basophils and mast cells also express a high-affinity receptor for IgE (FcεRI) which, when activated, induces release of histamine and other mediastors that typify allergic reactions.

Eosinophils.

Eosinophils are innate immune leukocytes classically associated with type 2 immune responses to allergic diseases and metazoan parasites such as *Nippostrongylus brasiliensis* (Shinkai et al. 2002; Voehringer et al. 2004). In cytologic and histologic preparations the cells have multilobulated nuclei, similar to neutrophils, but are dis-

tinguished from neutrophils by the characteristic eosinophilia (pink to red staining) of the cytoplasmic granules of eosinophils when stained with Romanowky-type cytologic stains or, to a lesser degree, by the routine hematoxylin and eosin (H&E) stain used in histopathology. Eosinophil infiltration into tissues has classically been associated with metazoan parasitism and allergic reactions, but recent evidence suggests eosinophils may have broader capabilities that include the regulation of inflammation, maintenance of epithelial barrier function, tissue remodeling, and bridging between innate and adaptive immunity (Shamri et al. 2011). Few eosinophils are present in the circulating blood, but greater numbers are located at mucosal surfaces that interface with the environment. In these locations eosinophils are well-positioned to interact with invading pathogens and, due to their content of pre-formed effector molecules, well-prepared to mount an immediate response.

Eosinophils have four types of cytoplasmic storage compartments: primary granules, secondary/specific/crystalloid granules, small amorphous granules and secretory vesicles (Egesten et al. 2001). Eosinophils also contain non-membrane-bound lipid bodies that contain the enzymes involved in production of eicosanoids. Eosinophil granules contain numerous pre-formed proteins, including more than 35 cytokines with T_H1, T_H2 and immunoregulatory capabilities, and four distinctive cationic proteins (Neves and Weller 2009). The specific granules of eosinophils contain two forms of major basic protein (MBP-1 and MBP-2), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and two ribonucleases (RNase2 and RNase3) (Scapini et al. 2013). MBP, which constitutes more than 50% of the eosinophil granule mass, lacks enzymatic activity but is highly cationic due to its content of 17 arginine residues (Popken-Harris et al. 1998). The cationic MBP binds to the plasma membrane of invading parasites, which changes the charge on the cell membrane, disorganizes the lipid bilayer, and increases permeability, thus resulting in toxic injury to the parasites (Hogan et al. 2008). MBP also causes much of the tissue injury seen with allergic diseases (Hogan et al. 2008; Shamri et al. 2011). EDN and the ribonucleases are toxic to metazoan parasites. EPO, which constitutes 25% of the eosinophil granule mass, catalyzes the oxidation of halides and nitric oxides to yield products that are toxic to both microbes/parasites and host cells (Thomas and Fishman 1986). The preformed proteins can be released very quickly in comparison to lymphocyte/macrophage secretion of immunologically active molecules, which typically is delayed by the processes of gene transcription, mRNA translation, and intracellular processing and packaging of proteins prior to secretion.

Eosinophils are a major source of lipid-derived inflammatory mediators such as cysteinyl leukotrienes (Bandeira-Melo and Weller 2003). The arachadonic acid metabolism that results in leukotriene formation in eosinophils takes place largely in cytoplasmic lipid bodies, which contain cyclooxygenases, 5-lipoxygenase, and leukotriene C_4-synthase (Bozza et al. 1997). The resultant cysteinyl leukotrienes have broad inflammation-associated effects such as bronchoconstriction, mucus production and increased venular permeability in asthma and allergic diseases.

Upon stimulation by TNF-α, eosinophils release a number of pro-angiogenic factors which result in the angiogenic processes that are prominent in eosinophil-associated diseases such as asthma (Hoshino et al. 2001a; Hoshino et al. 2001b).

In addition to direct opposition to metazoan parasitism and participation in allergic reactions, eosinophils express a number of proteinases and other enzymes that are involved in wound healing and tissue remodeling. Prominent among these are matrix metalloproteinase (MMP)9 and MMP17, which degrade extracellular matrix and enable cell migration through tissues (Dahlen et al. 1999; Gauthier et al. 2003). These products of eosinophils probably contribute to the infiltrative nature of mast cell neoplasms, which typically have an associated eosinophil population.

Eosinophils and mast cells are mutually supportive and have synergistic interactions. Chymase from mast cells recruits eosinophils to sites of inflammation, suppresses eosinophil apoptosis, and promotes the secretion of cytokines and chemokines by eosinophils (Wong et al. 2009). In reciprocity, eosinophils produce a stem cell factor that induces the activation, differentiation, maturation and survival of mast cells, probably via interactions that require direct contact between eosinophils and mast cells (Matsuba-Kitamura et al. 2010).

Effector functions of eosinophils require release of the pre-formed effector molecules that are stored primarily in cytoplasmic granules (Lacy and Moqbel 2000). Mast cells and basophils undergo acute exocytotic degranulation upon cross-linking of their Fcε receptors, with survival of the cells, but a similar mechanism of eosinophil degranulation has not been identified (Neves and Weller 2009). In contrast with mast cells and basophils, cross-linking of Fc receptors for IgG and IgA on eosinophils results in cytolysis of eosinophils, with release of cationic granule proteins as well as free membrane-bound granules from the dying eosinophils. Complete exocytosis of eosinophil granules is rarely seen *in vivo* except when eosinophils are in contact with the surface of large metazoan parasites.

The more common form of eosinophil granule release involves a process known as 'piecemeal degranulation'(Lacy and Moqbel 1997; Moqbel and Coughlin 2006). This process involves the formation of vesiculotubular carriers ('eosinophil sombrero vesicles') that fuse with the eosinophil plasma membrane, resulting in release of vesicle contents into the extracellular milieu (Melo et al. 2009). In addition, exteriorization of intact protein-laden cytoplasmic granules is known to occur in association with allergic diseases and parasite infestations. The extruded granules have receptors that receive signals from cytokines and chemokines, and release granule contents upon appropriate stimulation. In summation, the defensive proteins produced by eosinophils are pre-formed and available for immediate release from cytoplasmic granules, from previously extruded cytoplamic granules, and from previously released exosomal vesicles containing the defensive proteins.

Eosinophils also form extracellular 'traps' for bacteria, similar to neutrophilic extracellular traps (NETs) (Yousefi, 2009). Formation of the eosinophil-derived extracellular traps involves IL-5 or INF-γ priming of eosinophils and encounter with Gram-negative LPS, with subsequent release of mitochondrial DNA and granule-derived proteins. Formation of eosinophil extracellular traps is accomplished without death of the eosinophils.

The primary granules of human eosinophils also contain Charcot–Leyden crystal protein, which forms characteristic hexagonal bipyramidal crystals that can be detected microscopically in the stool or sputum of patients with enteric parasitism

or allergic pneumonitis (Egesten et al. 2001; Neves and Weller 2009; Welsh 1959). Similar crystals may be seen in association with eosinophil-mediated diseases of nonhuman primates (el-Hashimi 1971; Chalifoux and King 1983). Though debated for some time, it appears that Charcot–Leyden crystals may be seen in basophil-related disease processes of humans (Ackerman et al. 1982), thus may also be possible in basophil-related diseases of nonhuman primates.

In addition to innate immune functions, eosinophils have a number of functions in the adaptive immune system. Eosinophils traffic from sites of inflammation to draining lymph nodes and serve as APCs to preferentially promote T_H2 differentiation of T cells (Shi et al. 2000; MacKenzie et al. 2001; Padigel et al. 2006; Wang et al. 2007). Circulating eosinophils of humans constitutively store multiple cytokines with nominal T_H1, T_H2, and regulatory capacities, including IL-4, IL-13, IL-6, IL-10, IL-12, IFN-γ, and TNF-α (Spencer et al. 2009). Even though circulating eosinophils are armed with both T_H1 and T_H2 cytokines, they preferentially release T_H2-promoting IL-4 rather than T_H1-promoting IL-12 in response to polarizing stimuli that would be expected to elicit either a T_H1 or T_H2 response, thus are considered to be biased in favor of T_H2 responses.

Basophils.

Basophils, the rarest of the circulating leukocytes, are recognized by their lobulated nuclei and presence of cytoplasmic granules that are blue with Romanowsky-type cytologic stains and somewhat basophilic with standard H&E histology stains. Due to the paucity of basophilic cytoplasmic granules in their basophils, it was once thought that mice do not have basophils (Urbina et al. 1981), however, basophils are now routinely identified in mice by flow cytometry and immunohistochemistry (Schwartz and Voehringer 2014).

Based on the presence of basophilic cytoplasmic granules and surface expression of high-affinity IgE receptor on both cell types, basophils have historically been considered minor and perhaps redundant relatives of mast cells, or perhaps circulating precursors of mast cells (Karasuyama and Yamanishi 2014). This view was revised upon the discovery that basophils are a major source of the T_H2 cytokine IL-4, suggesting a role for basophils in allergy and defense against metazoan parasites (Karasuyama et al. 2011a).

Development of basophils in the bone marrow is closely related to mast cell development (Arinobu et al. 2005; Metcalf et al. 2013). Basophils develop in the bone marrow from a common granulocyte/monocyte precursor (GMP) which may also give rise to mast cells, depending on the sequence of expression the transcription factors c/EBPα and GATA2 (Iwasaki et al. 2006). C/EBPα and MITF are antagonistic transcription factors that silence each other, with C/EBPα expression leading to basophil differentiation and MITF expression leading to mast cell differentiation (Qi et al. 2013). Expression of Ikaros limits basophil development by limiting C/EBPα expression (Rao et al. 2013). Additional transcription factors such as P1-Runx1 and GATA-1 also have a role in basophil development (Mukai et al. 2012; Nei et al. 2013). There is evidence that development of basophils in humans may be somewhat different from the basophil developmental pathway in mice, as the human basophil developmental pathway includes immature basophils with basophil-

eosinophil hybrid phenotype (Grundstrom et al. 2012). A basophil/mast cell committed progenitor (BMCP) has also been described in the spleen of adult mice (Arinobu et al. 2005). Basophils leave the bone marrow as mature cells and have a circulating lifespan of approximately 60 h (Ohnmacht and Voehringer 2009).

IL-3 is the most important cytokine in driving basophil differentiation via the STAT5 signaling pathway, and IL-3-deficient mice generally do not develop basophilia in response to helminth infections (Lantz et al. 2008). Thymic stromal lymphopoietin (TSLP), another STAT5-activating cytokine, has also been shown to promote basophilia in the absence of IL-3 (Siracusa et al. 2011). There is somewhat conflicting data regarding the pathways that promote basophil differentiation and development, but STAT5 signaling is a consistent requirement (Eberle and Voehringer 2016).

Despite their uniform microscopic appearance, basophils are not a homogeneous cell population. Basophils can be subdivided into groups that are elicited by either IL-3 or thymic stromal lymphoprotein (TSLP). Basophils that develop in response to IL-3 tend to be involved in IgE-dependent adaptive immune responses, while the TSLP-dependent group tends to be involved in IgE-independent innate immune responses. TSLP-induced basophils are also involved in the pathogenesis of atopic dermatitis (Siracusa et al. 2011; Moniaga et al. 2013) and various forms of food allergy (Noti et al. 2014; Noti et al. 2013; Muto et al. 2014) as well as T_H2 responses to helminth infections (Giacomin et al. 2012).

Basophils express all the molecules that are necessary to function as APCs, including MHC class II, CD80, CD86, and CD40, and there is evidence that basophils are the critical APCs for driving T_H2 responses in mice (Sokol et al. 2009; Yoshimoto et al. 2009; Perrigoue et al. 2009). However, there is conflicting data regarding the full extent of this role for basophils in mice, and studies in humans have revealed no APC role for basophils (Karasuyama and Yamanishi 2014).

Basophils are conserved as a minor leukocyte population in many animal species, which suggests basophils have an important beneficial role in homeostasis. Basophils have a crucial role in defense against infestation by blood-sucking ticks (Wada et al. 2010), which are directly damaging to hosts and transmit numerous microbial pathogens. Tick infestation of mammals presents a unique set of immune system modifications that permit long-term cutaneous attachment and blood feeding. Ticks have developed countermeasures to inhibit virtually all facets of the innate and adaptive immune system, as well as anticoagulants that permit continual feeding on liquid blood at the attachment site. Interference with host immune resistance starts with hindrance of innate inflammatory cell infiltration into the site of tick attachment. Salivary gland extract of *Dermacentor andersoni* reduces endothelial cell expression of intracellular adhesion molecule-1 (ICAM-1), and a similar extract from *Ixodes scapularis* reduces expression of vascular cell adhesion molecule-1 (VCAM-1) and P selectin (Maxwell, 2005). Inflammatory cell influx is further impaired by the tick saliva content of disintegrin metalloprotease-like molecules which down-regulate the expression of β2 integrin molecules on the surface of neutrophils (Guo, 2009). All of these factors serve to reduce inflammatory cell emigration from blood vessels at the site of tick attachment.

Despite the resistance tactics displayed by ticks, mammalian hosts sometimes develop innate and adaptive immune responses that limit tick infestations following the initial infestation. The tick attachments sites of initial infestations typically contain few inflammatory cells, while the attachment sites of subsequent tick infestations often have numerous infiltrating inflammatory cells (Krause, 2009). Some tick infestations are characterized by basophil infiltration at the site of attachment (Allen, 1973, 1977), and basophils have been shown to have a non-redundant role in resistance to infestation by some tick species (Wada et al. 2010). Basophils are recruited to sites of tick feeding during the second, but not the first, infestation, indicating the first tick feeding transmits some type of 'information' to the basophil population. Experimental deletion of basophils results in loss of protection against the second tick feeding.

Basophil-mediated defense against helminths appears to be limited to effects on skin-invading larvae, as is seen with hookworm infestation, rather than a direct effect on adult helminths in the intestinal tract (Obata-Ninomiya et al. 2013). *Nippostrongylus brasiliensis*, a nematode parasite with a life cycle similar to the major human hookworm parasites, *Necator americanus* and *Ancylostoma duodenale,* has been used extensively to study immune resistance to helminth infection. The lifecycle of this parasite involves cutaneous penetration by larvae that subsequently migrate via the blood to the lung, are coughed up and swallowed to reach the final destination in the intestine. Basophils and eosinophils cooperate in trapping the third stage larva (L3) of *N brasiliensis* in the skin (Obata-Ninomiya et al. 2013). Many of the L3 larvae continue to migrate to the lung, where they are met by macrophages activated by the alternative pathway (Chen et al. 2014). Basophils are recruited to the intestine after the primary infection, resulting in a 10x increase in the basophil population which contributes to the rapid expulsion of the parasites (Schwartz et al. 2014). The basophils must be activated via surface-bound RcεRI and must secrete IL-4/IL-13 to expel the parasites. The final expulsion involves activation of goblet cells and other cell types in the intestinal mucosa.

In keeping with the general theme of basophil involvement to resistance to cutaneous parasite infection, basophils are also know to be involved in immunity to the migrating larvae that cause filariasis (Torrero et al. 2013), and are recruited to the skin of patients with cutaneous *Sarcoptes scabiei* infections (Ito et al. 2011).

Though resistance to parasite infection and contribution to allergic reactions would seem to be disparate functions of basophils, details of the reactions reveal many similarities. Helminth infection is characterized by increased numbers and/or activation of eosinophils, basophils, mast cells, T_H2 cells, and type 2 innate lymphoid cells (ILC2), as well as elevated serum levels of IgE (Eberle and Voehringer 2016). Allergic reactions are characterized by similar immune responses, causing some to suggest the immune system misinterprets allergens as helminthic parasites and mounts similar reactions in either disease process.

Basophils have a prominent involvement in various allergic inflammatory diseases, and commonly infiltrate into areas of allergic inflammation in the company of eosinophils and T_H2 lymphocytes (Stone et al. 2010; Karasuyama et al. 2011b). Basophils produce a large number of inflammatory mediators, many of which are

preformed, complexed with sulfated proteoglycans, and stored in cytoplasmic granules for immediate release upon appropriate stimulation (Stone et al. 2010). Basophils, along with eosinophils and mast cells, were long known to be essential components of IgE-mediated type I hypersensitivity reactions and allergic inflammation (Scapini et al. 2013). More recent evidence suggests basophils also participate in a variety of additional processes such as leukocyte recruitment, stromal cell activation, tissue remodeling, angiogenesis, and modulation of immune responses (Scapini et al. 2013). Basophils also serve to prolong allergic inflammation by release of histamine and LTC_4 as well as IL-4 and IL-13 (Karasuyama et al. 2011b; Sullivan and Locksley 2009; Schroeder 2011).

Chronic idiopathic urticaria of humans involves chronic cutaneous hives that are very irritating to patients, and have a significant effect on quality of life. The cutaneous lesions typically have basophil infiltration (Ying et al. 2002; Ito et al. 2011). An anti-IgE antibody (Omalizumab) significantly reduced clinical symptoms and, notably, and caused a reduction in basophil expression of FcεRI within 2 weeks of drug administration (Maurer et al. 2013). The combination of observations suggests IgE and basophils are involved in the pathogenesis of chronic idiopathic urticaria, and could be attractive pharmaceutical targets for this important human disease.

Basophils have a number pro-inflammatory roles, particularly in allergy and resistance to metazoan parasites, but are also known to have a regulatory role in allergic inflammation. In chronic allergic dermatitis, basophils secrete IL-4 that acts on recruited inflammatory monocytes to result in the generation of M2-type macrophages that dampen inflammation (Egawa et al. 2013). Basophil-origin IL-4 has also been shown to suppress inflammation in mouse models of arthritis (Campbell et al. 2014) and colitis (Gomez et al. 2014).

Basophils cooperate with dendritic cells in the promotion of T_H2 differentiation (Kumamoto et al. 2013; Otsuka et al. 2013; Tang et al. 2010) and present antigenic peptides to $CD4^+$ T cells (Otsuka et al. 2013). Basophils are also involved in the pathogenesis of the nephritis associated with lupus erythrematosis (Charles et al. 2010) and regulation of immune responses to bacterial pathogens (Bischof et al. 2014; Denzel et al. 2008).

See (Karasuyama and Yamanishi 2014) for a review of basophil involvement in immunity. See (Eberle and Voehringer 2016) for review of the role of basophils in protective immunity to parasitic infections.

Mast cells.

Mast cells were recognized by von Recklinghausen in 1863 and were named "mastzellen" by Paul Ehrlich in 1978 (Scapini et al. 2013). Subsequent studies focused on mast cell involvement in allergic reactions, and revealed mast cell production of histamine, IgE and slow-reacting substance of anaphylaxis, which was later shown to be leukotrienes. Mast cells are distributed throughout the mammalian body, with concentrations near blood vessels and host-environment interfaces such as skin and mucosal surfaces (Gurish and Boyce 2006; Bischoff 2007; Galli et al. 2008), where they constitute a first line of defense (Janssens et al. 2005; Marshall 2004). They are easily recognized in routinely stained histologic sections due to their content of basophilic cytoplasmic granules. Mast cells are particularly

abundant and prominent in various tissues of rats. The granules of mast cells, and basophils, exhibit the histochemical property of 'metachromasia', which means the granules display a purple color when stained with a blue dye such as toluidine blue. The metachromasia is a result of the granule contents of serglycin proteoglycans, which have a high anionic charge and selectively bind to cationic dyes (Ronnberg et al. 2012). Mast cells in tissue sections are definitively identified by immunohistochemical staining for tryptase or chymase, the former being preferred for rodent tissues and the latter being preferable for human tissues (Irani et al. 1989). It should be noted that 'tryptase' and 'chymase' are not specific chemical entities, but rather are collective terms for groups of serine endoproteases that have trypsin-like or chymotrypsin-like proteolytic activity, respectively. It is highly probable that multiple chemical entities are stained by primary IHC antibodies to the 'tryptase' or 'chymase' contents of mast cell granules, which raises significant questions regarding lot-to-lot antibody variability in IHC staining for mast cells.

Mast cells are subcategorized as serosal mast cells (SMC), connective tissue mast cells (CTMC), or mucosal mast cells (MMC) (Miller 1996; Enerback 1987; Befus 1986). Connective tissue mast cells are constitutively expressed and T cell-independent, while mucosal mast cells are induced and T cell dependent (Barrett and Austen 2009). There is evidence that mast cell phenotype is reversible under certain environmental conditions, and transdifferentiation between the phenotypes has been shown (Stone et al. 2010). MMC in rodents are morphologically and functionally atypical, with distinctive fixation requirements and histochemical properties as well as distinctive protease content of granules (Miller 1996; Enerback 1987). MMC pay key roles in airway and gastrointestinal diseases (Metcalfe et al. 1997), including asthma (Schwartz 1990), nematode infestations (Knight et al. 2000), stress-induced enteropathies (Castagliuolo et al. 1998), and reperfusion injury (Boros et al. 1995). Mast cells in humans have differential expression of tryptase or chymase activity in different tissues (Irani and Schwartz 1994), which may be regulated by the local environment in a tissue-specific manner (Miller 1996; Gibson et al. 1987; Stevens et al. 1994) as well as genetic makeup (Stevens et al. 1994; Ge et al. 2001).

Mast cells (MC) are derived from the bone marrow but final maturation takes place in peripheral tissues under the influence of local growth factors, particularly stem cell factor (SCF, also known as KIT ligand) and IL-3 (Gurish and Austen 2012). There is considerable debate regarding the lineage of MC, but current evidence suggests MC originate from bipotent progenitors in the granulocyte/monocyte progenitor (GMP) lineage (Dahlin and Hallgren 2015). The bipotent progenitors have the ability to develop into either mast cells or basophils. CCAAT/enhancer binding protein-α (C/EBPα) is the critical transcription factor for specifying basophil fate, while microphthalmia-associated transcription factor (MITF) is critical for specifying mast cell fate (Huang et al. 2016). C/EBPα and MITF silence each other's transcription in a directly antagonistic fashion. After commitment to the mast cell lineage in the bone marrow, mast cell precursors (MCp) circulate in the blood and home to peripheral tissues in an immature state. MCp contain few, if any, of the metachromatic cytoplasmic granules that characterize mature MC, therefore the

MCp are not identified by the histochemical stains that are routinely used to define MC in tissues. Increased MC population in tissues is thought to occur by recruitment of additional bone marrow MCp rather than local proliferation (Dahlin and Hallgren 2015).

The gastrointestinal tract has a special role in the development of mast cells. The intestine contains even more MCp than the bone marrow (Gurish 2001), and the population of MCp in the intestine is present in germ-free mice that have no antigenic stimulation from intestinal microbes (Guy-Grand, 1984). Studies have shown that MCp homing to the intestine is governed by expression of integrin α4β7 and its ligands (MAdCAM-1 and VCAM-1), as well as chemokine CXCR2 (Abonia et al. 2005).

Mast cells have a long life in tissues, which can continue after degranulation and re-granulation (Walker 1961; Kobayasi and Asboe-Hansen 1969; Xiang et al. 2001). Mature mast cells are generally considered to be terminally differentiated, with no capacity for replication. However, mature mast cells of mice have the ability to proliferate when injected into the skin of mast cell deficient mice (Sonoda et al. 1984), and human cutaneous mast cells have the potential for proliferation *in vitro* (Kambe et al. 2001). Despite these observations, the general consensus is that increases in mast cell populations in peripheral tissues result from recruitment of circulating MCp of bone marrow origin (Dahlin and Hallgren 2015).

The inflammatory mediators produced by mast cells are categorized as pre-formed mediators (which are stored in cytoplasmic granules), newly synthesized lipid-based mediators, and cytokines/chemokines (Wernersson and Pejler 2014). The pre-formed mediators include histamine and mast cell neutral proteases (tryptase, chymase, cathepsin G and carboxypeptidase), which are complexed with sulfated proteoglycans in the granules (Stone et al. 2010). Activated basophils and mast cells release both redundant and unique mediators, including histamine, proteoglycans, lipid mediators, proteases, chemokines and cytokines (Karasuyama et al. 2011a; Galli and Tsai 2010; Siracusa et al. 2013; Voehringer 2013). The cytoplasmic secretory granules of mast cells are rich in neutral serine endopeptidases (Miller and Pemberton 2002). More than 50 mast cell-derived endopeptidases in 11 animal species have been identified (Bairoch and Apweiler 2000), and the majority have trypsin-like (tryptase) or chymotrypsin-like (chymase) activities that are highly selective for different substrates. Lipid mediators produced by mast cells include platelet activating factor (PAF), prostaglandins and leukotrienes. These mediators serve to recruit neutrophils, eosinophils and basophils, cause bronchoconstriction, promote vascular permeability and induce mucus production (Scapini et al. 2013). Mast cells contain a number of cytokines and chemokines, and are considered the only cell with abundant storage of TNFα, which can be released immediately upon appropriate stimulation.

Mast cells, along with eosinophils and basophils, are critical elements of the IgE-mediated type I hypersensitivity (allergic) reaction. Mast cells are pre-positioned in tissues, thus are poised to mount an immediate response without the delay involved in recruitment of additional leukocytes, and certainly without the delay involved in *de novo* inflammatory mediator generation by cells of the adaptive immune system.

These responses contribute substantially to the immediate increase in vascular permeability and tissue swelling seen with IgE-mediated allergic reactions (Amin 2012). If the allergic inflammation persists, basophils, eosinophils and T_H2 lymphocytes are recruited to the site. Mast cells tend to form pro-inflammatory mediators in the early stage of inflammatory reactions, but switch to production of resolution-type molecules in the later phases of inflammation. Release of mast cell granule contents is similar to the process in basophils, consisting of fusion of granules with the plasma membrane and release of granule contents to the extracellular milieu.

In addition to their participation in allergic reactions, mast cells join basophils in contributing to leukocyte recruitment, stromal and tissue activation, modulation of immune responses, tissue remodeling and angiogenesis (Shelburne and Abraham 2011; Tsai et al. 2011). Some of the mast cell mediators of inflammation involve direct physical contact between mast cells and other cells of the immune system, e.g., contact between mast cells and B lymphocytes in areas of inflammation and in draining lymph nodes (Tsai et al. 2011; McLachlan et al. 2008). Concurrent administration of mast cell activators along with vaccines is known to result in a higher level of serum antibodies, therefore, the ability of activated mast cells to propel greater immunologic responses represents an attractive pharmaceutical target (McLachlan et al. 2008). Mast cells, as other innate immune cells, can form extracellular 'traps' that contain antimicrobial peptides, histones, DNA and tryptase (Tsai et al. 2011; Abraham and St John 2010). Mast cells express a number of PRRs, including numerous TLRs and NLRs, that can respond to pathogen-associated and danger-associated molecular signals (Shelburne and Abraham 2011).

Type 2 immunity, characterized by production of IL-4, IL-5 and IL-13, is highly involved in resistance to helminth parasite infections and the pathogenesis of allergic disease. These pathologic conditions affect billions of humans worldwide, resulting a major impact on human and animal health, with staggering health care costs. Type 2 effectors include IgE-producing B cells, $CD4^+$ type 2 helper (T_H2) cells, $CD8^+$ cytotoxic type 2 (T_C2) cells, type 2 innate lymphoid cells (ILC2), and the type 2 group of granulocytes that includes eosinophils, basophils and mast cells. Basophils and mast cells also have a critical role in immune regulation, autoimmune disease, and cancer (Huang et al. 2016). Basophils are very active IL-4 factories, producing more IL-4 than T_H2 lymphocytes on a per cell basis. Mast cells are said to be the most active chemical factories in the body, but produce very little IL-4. Both basophils and mast cells produce an abundance of IL-13, which induces allergic inflammation and, in conjunction with IL-4, is involved in expulsion of helminth parasites (Sullivan et al. 2011).

Current therapy of basophil- and mast cell-mediated diseases are aimed at the mediators produced by the cells but, as more information is obtained, interventions aimed at reducing or enhancing the differentiation or growth of basophils and mast cells may be more effective at achieving long-term clinical success (Huang et al. 2016).

Mast cells are sentinel immune cells that contribute to host defense against pathogens via innate immune systems, utilizing signaling and effector pathways such as TLRs and complement receptors (Voehringer 2013). IgE-mediated mast cell

activation is important in resistance to some parasites (Gurish et al. 2004). Mast cells also influence dendritic cells, macrophages, T cells and B cells, thus contributing indirectly to host defense (Abraham and St John 2010). Inappropriate or excessive mast cell activation contributes to allergic autoimmune reactions (Metz et al. 2007). All these activities result from positive influences of mast cells, but it is known that mast cells also have suppressive influences on immune reactions. Mast cells are essential intermediaries in development of regulatory T cell tolerance (Lu et al. 2006). Disruption of this regulatory axis may contribute to psoriasis and tumor growth/metastasis (Huang et al. 2016).

Mast cells accumulate at sites of allografts such as liver (El-Refaie and Burt 2005), kidney (Goto et al. 2002) and lung (Jungraithmayr 2015), suggesting that mast cells may contribute to allograft rejection. Following skin allografts, tolerogenic dendritic cells (DCs) in draining lymph nodes are important in the formation of Tregs, which contributes to allograft tolerance (Ochando et al. 2006; de Vries et al. 2011). Mast cells produce TNF and GM-CSF which are required for migration of DCs from skin grafts to draining lymph nodes to form the tolerogenic dendritic cells, thus absence of mast cell mediators hinders the generation of Tregs in the draining lymph nodes and impedes allograft tolerance.

Mast cells may contribute to acute graft-versus-host disease (GVHD) in humans (Jungraithmayr 2015), as evidenced by the suppression of GVHD by blocking the binding of IgE to FcεR1 (Korngold et al. 1997) and delayed onset of GFHD in mast cell-deficient mice (Murphy et al. 1994).

There are conflicting data regarding the involvement of mast cells in delayed-type hypersensitivity reactions and contact hypersensitivity, with some models showing involvement of mast cells and other models showing no such involvement. In those models showing a down-regulatory effect of mast cells, that effect is commonly manifested via mast cell influences on Tregs (Morita et al. 2016).

Activation of mast cells and basophils results in a positive feedback loop that must be controlled, otherwise an endless escalation of mast cell and basophil activation will occur. Adenosine 5′-triphosphate (ATP) is released from activated, dying or damaged cells, and the exteriorized ATP induces immune responses via the P2X and P2Y purinergic receptors that recognize ATP, UTP and ADP (Burnstock 2007; North 2002; Van Kolen and Slegers 2006). An abundance of ATP stored in the cytoplasmic granules of mast cells and basophils (Novak 2003; Gordon 1986; Bulanova and Bulfone-Paus 2010) is released by exocytosis following via cross-linking of FcεRI, as in allergic reactions (Nakanishi and Furuno 2008; Oury et al. 2006). The released ATP activates more basophils and mast cells in an autocrine fashion. Nucleotide-converting ectoenzymes such as ectonucleoside triphosphate diphosphohydrolase-1 (E-NTPD1), E-NTPD7, and ectonucleotide pyrophosphatase/phosphodiesterase-3 (E-NPP3) hydrolyze the released ATP, thus inhibiting ATP-dependent immune responses and contributing to immune response regulation. E-NPP3 is rapidly induced on basophils and mast cells as they are activated and hydrolyzes ATP on cell surfaces, thus preventing an endless escalating cycle of basophil and mast cell activation (Tsai and Takeda 2016). Pharmacological influences on this regulatory system would appear to have utility in the control of allergic

responses, but the possibility of a simultaneous undesirable effect on metazoan parasite immunity must be considered.

Mast cell infiltration is a negative prognostic feature in many forms of human cancer, and mast cells have an essential role in tumor progression in a number of mouse models of cancer (Khazaie et al. 2011). Much of the pro-tumor effect is based on mast cell promotion of angiogenesis and reorganization of the tumor microenvironment (Crivellato et al. 2008). The specificity of the mast cell effect is demonstrated by the tumor growth inhibition that occurs when mast cell degranulation is experimentally blocked (DeNardo et al. 2009; Samoszuk and Corwin 2003; Soucek et al. 2007). In addition to promoting angiogenesis, mast cells also modulate hemostasis and blood perfusion in tumors, largely due to the effects of heparin released from mast cells (Samoszuk et al. 2001).

In other forms of human cancer it appears that mast cells have a protective effect. Mast cell infiltration is a favorable prognostic feature in patients with colon cancer, non-small cell lung cancer, and B cell follicular lymphoma (Carlini et al. 2010; Ogino et al. 2009; Hedstrom et al. 2007), and is predictive of a more favorable outcome in melanoma patients that receive IL-2 therapy (Ali et al. 2009b; Ali et al. 2009a). In some of the clinical cases it appears the favorable versus unfavorable tumor progression is related to the enzyme content the infiltrating mast cells, with infiltration by tryptase-secreting mast cells being associated with poorer clinical prognosis (Kankkunen et al. 1997). Location of the infiltrating mast cells may be important in the ultimate influence on tumor progression. Intratumor mast cell infiltration in prostate cancer inhibits angiogenesis and tumor growth, while peritumor mast cell infiltration promotes tumor growth (Johansson et al. 2010). Presence of mast cells in the stroma of invasive breast cancer correlates with better prognosis (Dabiri et al. 2004; Rajput et al. 2008).

In addition to direct modulation of tumor progression, mast cells also influence tumor growth by indirect effects through the adaptive immune system. Mast cells are antigen-presenting cells, promote the migration, maturation and function of dendritic cells, and interact with both T and B cells (Khazaie et al. 2011). Recruitment of Treg into tumors is partially due to release of adenosine-containing molecules from mast cells (Huang et al. 2008). The positive or negative effect of mast cells on tumor progression is a reflection of the intracate and reciprocal interactions between mast cells and Tregs. The result of these interactions determines the level of cancer-associated inflammation, which may enhance or suppress tumor growth (Khazaie et al. 2011).

IL-10 is specific Treg signal that appears to be important in determining the positive or negative effects of mast cells on tumor growth and promotion. Loss of expression of IL-10 by Tregs is a hallmark change that indicates the Tregs have switched from an anti-inflammatory to pro-inflammatory phenotype (Asseman et al. 1999; Maloy et al. 2003; Erdman et al. 2003). If IL-10 is absent, as could occur with mast cell release of adenine-containing molecules into the tumor microenvironment, the transcriptional factor ICOS preferentially promotes the expression of IL-17A, a potent pro-inflammatory cytokine that promotes the growth and dissemination of colon cancer (Gounaris et al. 2009; Blatner et al. 2010). In summary, it

appears the presence of mast cell infiltration indirectly promotes a more pronounced inflammatory reaction that favors the growth and dissemination of colon cancer. This raises the possibility that pharmacologic modulation of mast cells or Tregs, or adoptive transfer of healthy Tregs, may alter the tumor microenvironment and influence clinical outcomes of colon cancer.

The involvement of mast cells, eosinophils and basophils in allergic reactions, which affect 20–30% of the human population, has led to the general concept that these cells have predominantly negative effects on the host. Involvement of these cells in resistance to metazoan parasite infection is not as widely appreciated, particularly in populations of advanced nations that have less exposure to these pathogens. There is a question of the evolutionary advantage of allergic reactions, which have universally negative and sometimes fatal consequences. Closer examination of the signaling and effector pathways of allergic reactions reveals similarity to defenses against metazoan parasites and, perhaps more precisely, to defenses against the venoms of various species (Profet 1991; Stebbings 1974). The type 2 immune responses to allergens, which are widely viewed as "misdirected" or "maladaptive" in nature (Holgate and Polosa 2008; Artis et al. 2012), may be the consequence of evolutionarily conserved but inappropriately applied defenses against these venoms (Galli et al. 2016).

2.2.1.4 NK & NKT Cells

NK cells are bone marrow-derived mononuclear cells that have markers of both T lymphocytes and macrophages. NK cells are somewhat larger than typical T cells, and a characteristic microscopic feature is the presence of distinct azurophilic cytoplasmic granules, thus the classical name of 'large granule leukocyte'. The azurophilic granules correspond to the osmiophilic granules seen via electron microscopy (Bouwens et al. 1987). The cytoplasmic granules have been shown to contain perforin and granzymes, which are involved in cell membrane attack and induction of apoptosis in target cells. Recognition of target cells by NK cells is not restricted to major histocompatibility complex (MHC) antigen presentation, as is the case with target recognition by cytotoxic T lymphocytes. Not only is MHC presentation of antigens not required, but NK cells selectively kill cells (such as tumor cells) that have a deficient level of MHC surface presentation. NK cell killing does not involve immunological memory, as it is classically known in the adaptive immune system, though there is emerging evidence that NK cells involved in pathologic processes may develop epigenetic modifications that enhance their responsiveness upon second exposure to the pathogen, thus exhibiting 'trained immunity' (Netea et al. 2015; Netea et al. 2016). NK cells exert antitumor effects by exocytosis of perforin/granzyme-containing granules, induction of apoptosis in target cells, and production of various cytokines that augment the functions of other immune cells (Nakatani et al. 2004).

Granzymes are the effector molecules in perforin/granzyme-mediated apoptosis, thus considerable attention has been given to details of granzyme activity and

modulation of that activity. Available evidence suggests granzymes have multiple pro-apoptotic effects on the apoptosis cascade. Mitochrondria are thought to be involved in at least one pathway of granzyme B-associated apoptosis induction, as granzyme B and perforin cause the release of cytochrome c into the cytosol before the activation of apoptosis. Granzyme B-induced apoptosis is highly amplified by mitochrondria in a caspase-dependent manner, but granzyme B can also initiate caspase 3 processing and apoptosis in the absence of mitochrondria (MacDonald et al. 1999).

NKT cells are regulatory T lineage cells that have a range of immune functions, including promotion of cell-mediated immunity to tumors, viruses and bacteria; preventing autoimmune disease through regulation of self-tolerance; and contributing to allergic diseases such as psoriasis and asthma (Bendelac et al. 2007; Salio et al. 2014). NKT cells have many of the phenotypic and physiologic characteristics of NK cells, but have the additional feature of surface expression of T cell receptor (TCR). The more common Type 1 NKT cells express a semi-invariant TCR composed of an invariant TCRα chain and a restricted assortment of TCRβ chains TCR (Rossjohn et al. 2012), while type 2 NKT cells express a more diverse TCR repertoire (Godfrey et al. 2004; Godfrey et al. 2010). The TCR on type 1 NKT cells is reactive to the glycolipid antigen α-galactosylceramide (αGalCer) (Van Kaer 2005; Van Kaer and Joyce 2005).

The TCR on NKT cells interacts with CD1d, a nonclassical member of the MHC family, as opposed to the TCR:MHC-class I/II interaction of T lymphocytes (Godfrey et al. 2010; Godfrey et al. 2004). CD1d is primarily involved in expression of lipid-type antigens. In the adaptive immune system, antigen processing into either MHC-I or MHC-II context involves time-consuming steps for antigen processing and molecule transposition to the cell surface. By contrast, constitutive expression of CD1d on cell surfaces allows NKT cells to interact with target cells without delay, thus NKT cells may be considered part of the 'rapid response team' of the innate immune response even though the cells are classified as lymphocytes.

NKT cells are thought to be involved in a number of human diseases, including various forms of cancer (Berzofsky and Terabe 2008; Vivier et al. 2012; Dhodapkar 2009), type 1 diabetes (Novak et al. 2007; Fletcher and Baxter 2009; Lehuen et al. 2010), and asthma (Kim et al. 2010; Holtzman 2012; Umetsu and Dekruyff 2010).

See (Berzins and Ritchie 2014) for review of NKT cell involvement in human diseases.

2.2.1.5 Innate Lymphoid Cells (MAIT Cells and iNKT Cells)

A number of different terms have been applied to innate lymphoid cells (ILCs), making it difficult to interpret published reports on various members of the ILC group. It has been proposed (Spits et al. 2013) that ILCs should be classified on the basis of their phenotypical and functional characteristics. This classification system, which is similar to that used for T_H cells, subdivides ILCs into three groups. Group 1 ILCs are those cells that produce IFNγ, with NK cells being the prototypical

member of the group. Group 2 ILCs produce type 2 cytokines (including IL-5 and IL13), require IL-7 for their development, and depend on GATA-binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα) for their development and function. Group 2 ILCs includes cells that have been previously described as natural helper cells, nuocytes and I_H2 cells. Group 3 ILCs produce IL-17 and/or IL-22, and depend on the transcription factor RORγt for their development and function. The prototypical ILC Group 3 cells are the LTi cells which are involved in formation of secondary lymphoid organs during embryogenesis and tertiary lymphoid tissue that is associated with inflammation.

Cells of the innate immune system are known for their ability to mount an immediate response to pathogen or danger signals, which is in contrast to the delayed response of the adaptive immune. A population of innate T cells serves during the lag phase as the adaptive immune response goes through the processes of antigen processing and presentation followed by T and B cell activation and proliferation. Innate T cells have somatically rearranged TCRs that undergo thymic selection but, unlike conventional T cells, the innate T cells recognize non-peptide PAMPs or DAMPs (Lawson 2012). Innate T cells include a group known as mucosa-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells (Beckman et al. 1994; Tilloy et al. 1999; Porcelli et al. 1993). The TCR of iNKT cells recognizes lipid-based ligands presented by CD1d molecules (Beckman et al. 1994). The TCR of MAIT cells recognizes a novel class of microbial molecular patterns that are derived from vitamin B-based metabolites, such as riboflavin, which are presented by MHC-related protein 1 (MR1), a nonpolymorphic class Ib MHC molecule (Corbett et al. 2014; Kjer-Nielsen et al. 2012)). Since mammals lack the capacity to synthesize riboflavin, intermediates from the riboflavin biosynthetic pathway are distinct microbial molecular patterns that provide a unique signal to the immune system.

Most MAIT cells are CD8a phenotype, with a minority of being $CD4^-CD8^-$ and essentially none being $CD4^+$. Cellular markers of MAIT cells suggest they are similar to IL-17-producing cells. MAIT cells make up 1–4% of the circulating TCR-αβ T cells in humans (Martin et al. 2009), and can constitute 50% of the T cells in the liver (Dusseaux et al. 2011). MAIT cell development is dependent on presence of intestinal microflora. Studies of MAIT cells in patients with ulcerative colitis (UC) and Crohn's disease (CD) have shown that circulating MAIT cell populations are reduced and mucosal MAIT cell populations are increased in UC and CD patients as compared to healthy subjects, and the mucosal MAIT cells appear to be activated in the disease state (Serriari et al. 2014).

2.2.1.6 Myeloid Suppressor Cells

The immune system has developed multiple systems for limiting immune reactions, which may be damaging to the host if they continue to an extreme degree or occur in an inappropriate location (Berzofsky et al. 2004). These control mechanisms largely involve generation or expansion of cell populations that negatively influence

T cell functions (Serafini et al. 2006). Myeloid suppressor cells (MSCs) serve to inactivate both $CD4^+$ and $CD8^+$ T cells in both neoplastic and non-neoplastic diseases (Bronte et al. 2001; Serafini et al. 2004; Kusmartsev and Gabrilovich 2002). The MSC population is a functional group of cells that includes immature macrophages, granulocytes, DCs and other myeloid progenitor cells. Neoplasms produce an incompletely defined group of cytokines, chemokines and other soluble molecules that can induce MSC recruitment and promote their maturation into immunosuppressive cells. MSC suppressive activity is largely regulated by arginine metabolism, specifically through the activities of the enzymes iNOS and ARG1 (Serafini et al. 2004; Bronte et al. 2003). Pharmacological interception of these arginine-related pathways may serve to hinder tumor-related promotion of myeloid suppressor cells, thus allowing normal immunologic resistance to the tumors (Serafini et al. 2006).

2.2.2 Cells of the Adaptive Immune System

Lymphocytes are the major functional cells of the adaptive immune system, and constitute a major component of circulating leukocytes. The relative percentage of circulating lymphocytes and neutrophils is a species-specific characteristic, with neutrophils predominating in humans, nonhuman primates, and dogs, and lymphocytes predominating in mice, rats, cattle and sheep. Lymphocytes are broadly grouped into B cells, based on origin in the bone marrow of mammals or the bursa of Fabricius of birds, or T cells, named because of their maturation in the thymus. Natural killer (NK) cells and NKT cells are additional types of lymphocytes that are discussed under the innate immune system.

The various types of lymphocytes cannot be distinguished by their microscopic appearance in either histologic sections or cytologic preparations. Definitive identification of lymphocytes and subtypes is based on demonstration of specific markers by immunohistochemistry or flow cytometry. These procedures can be used to further subcategorize lymphocytes and determine various features of activation or effector status.

Both B and T cells have multiple copies of surface receptors for specific antigens, all of which are specific for the same antigen. The antigen receptor for B cells is surface-bound immunoglobulin (sIg), which can bind directly to soluble or particulate antigens. By contrast, the antigen receptor for T cells is included in the T cell receptor complex, and can react to antigen only when the antigen is presented in association with the major histocompatibility complex (MHC) proteins on antigen-presenting cells. When activated, the lymphocytes multiply to form clones of cells, all of which express receptors for the same antigen.

Lymphocytes develop from hematopoietic stem cells (HSCs) in the bone marrow, which differentiate into multipotent progenitors (MPPs). A subset of MPPs become lymphoid-primed multipotent progenitors (LMPPs) that subsequently become common lymphoid progenitors (CLPs) (Love and Bhandoola 2011).

Development of lymphocytes is heavily dependent on Notch signaling, which influences multiple lineage 'decisions' of developing lymphoid and myeloid cells (Yuan et al. 2010; Radtke et al. 2010). The Notch family consists of four Notch receptors (Notch1–Notch4) and five ligands (Delta-like ligand 1 (DLL1), DLL3, DLL4, Jagged1 and Jagged 2. Notch signaling is activated at various stages of immune cell development, such as commitment to T cell versus B cell lineage, T cell differentiation into αβ versus γδ T cells, and differentiation into $CD4^+$ or $CD8^+$ single-positive T cells (Yuan et al. 2010; Radtke et al. 2010). Notch signaling is also involved in T cell-mediated immune responses such as the differentiation and function of cytotoxic and helper T cells (Amsen et al. 2009). Pathogen-associated molecular patterns promote expression of Notch ligands on the surface of antigen-presenting cells. Activation of naïve $CD8^+$ T cells requires binding of the DLL1 ligand on APC by Notch1 or Notch2, which leads to expression of multiple T_C signaling and effector molecules (*Eomes* > Eomes transcription factor; *Gzmb* > serine protease granzyme B; *Ifng* > interferon-γ; and *Prf1* > perforin 1) (Cho et al. 2009; Maekawa et al. 2003). In naïve $CD4^+$ T cells, ligation of DLL1 and DLL4 activate Notch signalling and transcription of *Tbx21*, which encodes T-bet (T_H1 cell transcriptional regulator) (Maekawa et al. 2003; Minter et al. 2005). During development of T_H2 cells, activation of Notch1 and Notch 2 by Jagged1 and Jagged2 favors the expression of *Gata3* (encoding transcription factor GATA-3) and *Il4* (encoding interleukin 4) (Amsen et al. 2004; Tu et al. 2005; Fang et al. 2007). Notch1 signaling is involved in differentiation of T_H17 and T_H9 subsets by promoting the expresion of *Rorc* (encoding transcription factor Rorγτ) and *Il9* (encoding interleukin 9) (Elyaman et al. 2012; Keerthivasan et al. 2011; Mukherjee et al. 2009). Notch signaling also has a critical function in controlling peripheral Treg cell function. Due to these diverse involvements in lymphocyte development and function, the Notch signaling pathway is an attractive pharmaceutical target.

2.2.2.1 T Lymphocytes

The thymus is the primary site of T cell development in jawed vertebrates, therefore, thymic structure and function are critical in the differentiation and selection of immunologically competent T cells (Miller 1961; Bevan 1977; Zinkernagel 1978). The thymus does not contain self-renewing hematopoietic stem cells, instead relying on bone marrow-derived progenitor cells that travel via the bloodstream (Wallis et al. 1975) and enter the thymus at the corticomedullary junction (Lind et al. 2001; Ceredig and Schreyer 1984). In order to complete their maturation, bone marrow-derived T cell precursors must go through a sequence of developmental steps that include a carefully orchestrated movement of cells through the thymic cortex and medulla (Love and Bhandoola 2011; Bhandoola et al. 2007). This sequence of events includes entry of progenitor T cells into the thymus, generation of $CD4^+$ $CD8^+$ (double-positive, DP) cells in the thymic cortex, positive and negative selection of DP thymocytes, interaction of positively selected thymocytes with medullary epithelial cells to complete development and insure central tolerance, and

export of mature T cells from the thymus (Sainte-Marie and Leblond 1964; Cantor and Weissman 1976; Stutman 1978; Bhan et al. 1980; Petrie 2003; Gray 2005). These processes involve physical movements of developing T cells in addition to evolving cellular maturation. After entering the thymus near the corticomedullary junction, the precursor T cells migrate up into the overlying cortex to start the initial phases of development. Later there is a reverse migration back to the deep cortex, with eventual maturation and release from the thymic medulla. It is estimated that fewer than 100 precursor cells/day reach the thymus of a young adult mouse per day but, over a period of approximately 2 weeks, this modest cell population becomes committed to T cell lineage, undergoes TCR gene rearrangement and cell surface expression of TCR, and undergoes a population expansion to approximately 50 × 10^6 cells. The response of the naïve T cell is remarkably rapid and robust. A naïve $CD8^+$ T cell can undergo 15 divisions to produce more than 10^4 progeny cells within 7 days of antigenic stimulation, at some stages of the response dividing every 4–8 h (Murali-Krishna et al. 1998; Doherty 1998; Callan et al. 1998; Butz and Bevan 1998). Thymic stromal cells provide 'instructions' for the developing T cells, but the information exchange is a two-way street in which the developing T cells also provide feedback instruction to the thymic stromal cells (Shores et al. 1991). This developmental and migratory pathway is fraught with danger for the developing thymocytes, and only 1–3% survive to exit the thymus (Scollay et al. 1980; Egerton et al. 1990; Goldrath and Bevan 1999).

The generation of mature (but immunologically naïve) T cells in the thymus has been the subject of intense investigation. Entry of progenitor cells is an intermittent, gated event that occurs in waves at intervals of 3–5 weeks in mice (Foss et al. 2001; Le Douarin and Jotereau 1975; Havran and Allison 1988). Lymphoid progenitor cells that enter the thymus during embryogenesis give rise to populations of γδ T cells, as opposed to the predominant αβ T cell populations that result from entry of lymphoid progenitor cells in adults (Havran and Allison 1988; Coltey 1989; Dunon 1997; Ikuta 1990; Weber-Arden et al. 2000). Thymocyte development continues in the cortex though the double-negative (DN)1 and DN2 stages, and reach a developmental checkpoint at the DN3 stage ($CD4^-$ $CD8^-CD25^+CD44^-$ cells) (Pearse 1989; Shinkai 1992). Only cells that succeed in TCR β chain gene rearrangement, known as the β-selection checkpoint, progress beyond the DN3 stage. Development up through the DN3 stage is controlled by Notch/Delta ligand signaling from cortical thymic epithelial cells (cTECs) (von Freedem-Jeffry 1995; Peschon 1994). Simultaneously, the thymocyte development regulates the differentiation of TEC precursor cells into mature cTECs (Klug 1998).

Concurrently with their cellular differentiation, the developing DN thymocytes migrate outward to the periphery of the thymic cortex (Lind et al. 2001). During this migration the developing thymocytes complete genetic rearrangements that allow cell surface expression of TCRβ and pre-TCRα in the pre-TCR complex (Raulet et al. 1985; von Boehmer and Fehling 1997). Expression of the pre-TCR complex along with Delta-Notch signaling allows the developing thymocytes to become double-positive (DP) thymocytes which express TCRαβ (von Boehmer and Fehling 1997; Irving et al. 1998; Ciofani and Zúñiga-Pflücker 2005). Expression of

the TCRαβ complex constitutes the first checkpoint mentioned above, which must be successfully negotiated in order for the developing thymocytes to progress to the DP stage.

Newly generated DP thymocytes express low levels of TCRαβ, which interact with MHC-peptide complexes expressed by cTECs and dendritic cells in the thymic cortex (Bousso et al. 2002). Low-avidity interactions between TCR and MHC-peptide complexes cause the DP thymocytes to receive further developmental signals that allow them to progress to single-positive (SP) thymocytes, a process known as positive selection. High-avidity interactions between TCR and MHC-peptide complexes results in apoptosis of the thymocytes, a process known at negative selection (Takahama 2006). In addition to the thymocytes that die of apoptosis due to negative selection, a major percentage of the developing thymocytes receive no TCR signal and also undergo apoptosis (Egerton et al. 1990; Goldrath and Bevan 1999). The DP thymocyte population is exquisitely sensitive to glucocorticoid-mediated apoptosis, which may be seen with systemic stress (Boldizsar et al. 2006).

Only cells that have a low level of MHC self-recognition and heterodimeric receptors are allowed to pass the second checkpoint (positive selection checkpoint). Cells that fail to survive the relatively narrow window (approximately 3 days duration) of positive and negative selection are doomed to 'die of neglect', and are promptly phagocytized by tingible body macrophages in the thymic cortex. Positively selected DP thymocytes then relocate from the cortex to the medulla, largely as a result of CCR7-mediated chemotaxis (Ueno 2004), with a possible contribution from the flow of interstitial fluid from the cortex toward the medulla (Eggli et al. 1986; Nieuwenhuis 1988). As another example of two-way communication in the thymus, migration of the DP thymocytes into the medulla serves to shape the medullary environment (Shores et al. 1991, 1994; Nasreen et al. 2003). The SP thymocytes spend approximately 12 days in the medulla, where they undergo further maturation to become naïve T cells (Reichert et al. 1986; Bendelac et al. 1992; Ramsdell et al. 1991). The newly generated SP thymocyte population is also highly sensitive to dexamethasone-induced apoptosis but, following maturation in the medulla, the mature (but naïve) T cells are dexamethasone-resistant (Takahama 2006). Thymocyte maturation in the medulla is important in the establishment of central tolerance to self antigens (Kyewski and Derbinski 2004). Expression of tissue-specific self-antigens by mTECs is partially dependent on the transcriptional factor known as autoimmune regulator (AIRE) (Zuklys 2000; Derbinski 2005), deficiency of which results in autoimmune diseases in humans and mice (Nagamine 1997; Aaltonen 1997; Anderson 2002; Liston et al. 2003; Kuroda 2005).

Entry of progenitor cells into the thymus and their subsequent maturation are two separate events (Foss et al. 2001, 2002). The 'thymus gate' permits progenitor cell entry in cycles with a periodicity of approximately 15 days, and progenitor cells that have entered the thymus begin to differentiate only when the previous progenitor cell cohort has exited or been depleted (Ceredig and Rolink 2002). Different clusters of thymocytes within the thymic cortex appear to be in a synchronized stage of development, which is manifested microscopically as clustering of apoptotic cells following experimentally administered glucocorticoids.

The size of the thymus, which is substantially smaller than the total bone marrow space, may limit the number of niches available for expansion of T cell populations after progenitor cells reach the thymus (Ceredig and Rolink 2002).

Flow cytometric analysis using CD4 and CD8 monoclonal antibodies reveals ~5% of thymocytes express neither CD4 nor CD8 (double-negative, DN cells), ~80% express both CD4 and CD8 (double-positive, DP cells), ~10% express only CD4 (CD4 single-positive, CD4SP), and ~5% express only CD8 (CD8SP)(Ceredig et al. 1983). These populations appear sequentially during thymocyte development. Flow cytometric analysis using CD3 monoclonal antibodies reveals the DN thymocyte population has $CD3^+$ and $CD3^-$ subsets (Bluestone et al. 1987). In addition to the conventional population of αβ T cells, the $CD3^+$ thymocyte population contains $CD3^+$ γδ (Havran and Allison 1988) and $CD3^{dull}$ $TCR\alpha\beta^+$ $NK1.1^+$ cells (natural killer T, NKT) (MacDonald 2002). The DN thymocyte population that also lacks CD3 expression has been termed the 'triple negative' (TN) population (Godfrey et al. 1992).

Newly formed naïve T cells undergo final maturation in the medulla before exiting the thymus and seeding peripheral lymphoid organs (Scollay and Godfrey 1995). Exit of mature thymocytes from the thymus is thought to occur through the perivascular space, but it is unclear whether exit is via venules, lymphatics, or a combination of these vessels.

Functions of both B and T cells start with antigen recognition. With B cells the antigen recognition is accomplished by the surface expression of specific antibody, but T cells have a more complex antigen receptor complex. Generation of the T cell receptor (TCR) complex involves a number of checkpoints that largely prevent the generation of TCRs that recognize self-antigens. These repertoire selection checkpoints are major steps in T cell development, and often constitute branch points for development of the various T cell subsets.

All jawed vertebrates have TCR complexes composed of either αβ heterodimers or γδ heterodimers, all of which are proteins of the immunoglobulin superfamily. Individual T cells have one or the other TCR complex, but not both. The TCR component molecules are type 1 transmembrane proteins, which are single-pass proteins with an extracellular N terminus. Antigen recognition is accomplished via highly variable regions in the external component of the molecules, which result from somatic mutation and recombination of V and D gene segments (α and γ chains) or V, D and J chains (β and δ chains) (Jenkins 2013). Each T cell typically expresses only a single type of α/γ or β/δ chain. The TCR complex also includes CD3γ, δ, and ε as well as TCRζ (or CD3ζ). These latter molecules have signaling functions but do not confer antigen specificity. TCR engagement with antigenic peptides held in MHC class I or class II molecules on antigen-presenting cells is facilitated by CD4 and CD8 co-receptors that interact with the most conserved segments of the MHC class I and MHC class II molecules, respectively. CD4 is a linear, monomeric member of the immunoglobulin superfamily, while CD8 is either homodimeric (CD8αα) or heterodimeric (CD8αβ).

The cytoplasmic domains of both CD4 and CD8 provide docking sites for Lck, a Src-family tyrosine kinase that plays a key role in T cell signaling. Lck-generated

signaling proceeds via numerous mediators, including calcium mediators and the PI3-kinase, Ras/MAP kinase and NF-κB pathways. Other intracellular signaling pathways impact on these major pathways to nudge T cell functions in various directions. In addition to these intracellular signal enhancers, there are signal influences from additional surface receptors not directly associated with the TCR complex. CD28 is an additional cell surface signaling molecule that serves to amplify TCR-mediated signaling, while CD5 is a cell surface signaling molecule that tends to dampen TCR-mediated signaling. CD28 has been a particularly attractive pharmaceutical target.

$CD4^+$ T cells respond to antigenic stimulation primarily by increased expression of IL-2, a growth factor. By contrast, $CD8^+$ T cells respond by increased production of perforin, which punches holes in plasma membranes, granzymes A and B, toxic molecules that are introduced through the holes punched by perforin, and toxic cytokines such as IFNγ. In addition to the basic $CD4^+$ and $CD8^+$ subdivision, there are multiple T cell subsets such as T_H1, T_H2, T_H17, T follicular helper, Treg, etc., that further define T cell functions. The various subsets of T cells have firm commitment to production of specific signaling and effector molecules which are elaborated upon antigenic stimulation. Between proliferative events, these characteristics of T cell subsets are maintained by transcription factor expression patterns and specific chromatin modifications. Thus Runx3, T-bet and eomesodermin maintain killer function, T-bet maintains T_H1 function, GATA-3 promotes T_H2 function, PLZF promotes NKT-cell function, RORγT promotes T_H17 function, Bcl6 promotes follicular-helper cell function, and Foxp3 promotes Treg function (Rothenberg and Champhekar 2013).

Naïve T cell populations specific for a specific antigen exist at a frequency of approximately 1:200,000 in the pre-immune repertoire (Jenkins et al. 2010). This equates to approximately 7×10^7 or 3×10^{11} naïve T cells in the body of an adult mouse or human, respectively. Naïve T cells have a circulating half-life of 2–5 years in humans and 50–100 days in mice (Hellerstein et al. 2003; Hataye et al. 2006). During this time they enter lymph nodes and mucosa-associated lymphoid tissue via high-endothelial venules (HEV), eventually reaching T-dependent areas such as the paracortex of lymph nodes. Similarly, naïve T cells enter the spleen via the marginal zone sinuses and migrate into the T-dependent periarteriolar lymphoid sheaths. In either the paracortex of lymph nodes or periarteriolar lymphoid sheaths of the spleen, the naïve T cells are retained in the regions by their expression of CCR7, which senses CCL19 and CCL20 (Cyster 2005; Jenkins 2013). Naïve T cells are excluded from B-cell-rich follicles in all secondary lymphoid tissue by their lack of expression of CXCR5, which binds to the CXCL13 that is produced in follicles and guides naïve B cells to this location.

The T-dependent areas of lymph nodes have a network of conduits that carry lymph-borne antigens and chemokines from the afferent lymphatics and subcapsular sinuses into sinuses surrounding the HEV. The conduits are surrounded by fibroblastic reticular cells that produce IL-7, a survival factor for naïve T cells (Link et al. 2007). Naïve T cells entering lymph nodes via HEV migrate along the conduits, receiving IL-7 support as they spend approximately one day in the lymph node in

their search for cognate antigen. In order to survive, naïve T cells must also receive TCR signals through weak recognition of p:MHC ligands, the protein recognition elements expressed by all nucleated cells. Dendritic cells probably provide this p:MHC ligand exposure to naïve T cells. Detection of p:MHC ligands by naïve T cells does not result in expression of the full complement of downstream signaling molecules that results from recognition of foreign antigens in MHC context, and does not result in proliferation of the naïve T cells. Competition for available IL-7 is a major factor in controlling the abundance of the naïve T cell population. When the naïve T cell population is low e.g., in neonates or following chemotherapy or irradiation, IL-7 produced by the fibroblastic reticular cells is relatively more available to the remaining naïve T cells and they are able to proliferate. This homeostatic proliferation of naïve T cells in secondary lymphoid organs is different from the response to foreign antigens in that CD28 co-stimulation is not required for the homeostatic proliferation of naïve T cell (Prlic et al. 2001).

Both survival and proliferation contribute to the number of naïve T cells in secondary lymphoid organs. When the thymus exports new naïve T cells to secondary lymphoid organs that already have a full complement of T cells, the newly arrived naïve T cells survive in interphase until the organ reaches a status where additional naïve T cells are required. When the newly arrived naïve T cells enter a secondary lymphoid organ that is sparsely populated with T cells, e.g., a neonate or (perhaps) aged individual, there is a relative abundance of IL-7 that permits population expansion of the newly arrived naïve T cells.

Subsets of effector $CD4^+$ T cells become 'polarized' to different functions depending on exposure to various cytokines during their development. Effector cells generated in the presence of IL-12, IL-4, IL-6 and TGF-β, or IL-6 and IL-21 become IFN-γ-producing T_H1 cells, IL-4-producing T_H2 cells, IL-17-producing T_H17 cells, or IL-21-producing follicular helper cells respectively (Jenkins 2013), which assume specific roles in resistance to intracellular microbes, metazoan parasites, or extracellular microbes.

As with $CD4^+$ helper T cells, $CD8^+$ T cells require costimulatory signals from innate immune cells to reach their full effectiveness as cytotoxic T lymphocytes (CTLs). Naïve $CD8^+$ T cells that receive antigenic stimulation via TCR/MHC class I ('first signal') also require signaling from CD28 ('second signal) and either IL-12 or type 1 interferon ('third signal') for development of cytolytic activity (Curtsinger and Mescher 2010). Dendritic cells activated by PRRs, or $CD4^+$ T_H cells complete with CD40-CD40L interaction, produce the requisite third signals that allow $CD8^+$ T cells to become functional CTLs.

The preponderance of T cell functions are associated with T cells that have α and β TCR chains, but there is an additional population of T cells that have γ and δ TCR chains. In some ways γδ T cells function as innate immune cells or non-conventional T cells, with several innate-like features that allow γδ T cells to undergo early activation following direct recognition of conserved non-peptide antigens that are upregulated by stressed cells, similar to the recognition of PAMPs and DAMPs by PRRs (Bonneville et al. 2010). The γδ T cell population arises early in ontogeny (GD 14 to GD 18 in mice), prior to development of the conventional

αβ T cell population (Tonegawa et al. 1989; Raulet 1989). The γδ T cells acquire a pre-activated status early in their development, which allows rapid induction of effector functions following detection of tissue stress. This rapid response capability, coupled with the common location of γδ T cells in epithelial surfaces such as skin and mucosa of the gastrointestinal, respiratory and reproductive systems, allow γδ T cells to serve as an effective early response team.

The Fas/FasL pathway and the perforin-granzyme system are utilized by γδ T cells to kill infected, activated or transformed cells (Qin et al. 2009; Dieli et al. 2001). Products of γδ T cells include directly bacteriostatic or lytic molecules such as granulysin and defensins (Dieli et al. 2001; Dudal et al. 2006). The γδ T cells induce antibacterial functions of other immune effector cells and epithelial cells (Hamada et al. 2008), including cytokines that are involved in immunity against viruses and intracellular pathogens (TNF and IFNγ), extracellular bacteria and fungi (IL-17), and extracellular parasites (IL-4, IL-5 and IL-13) (Bonneville et al. 2010). In addition to pro-inflammatory cytokines and epithelial growth factors, γδ T cells also produce down-regulatory cytokines such as TGFβ and IL-10 (Jameson and Havran 2007; O'Brien et al. 2007).

Innate immune responses typically precede and in some ways instruct adaptive immune responses, but some reverse interactions also occur. The reverse interactions commonly involve non-conventional T cells such as invariant NKT cells, which are αβ T cells restricted to the MHC class I-like CD1d molecule, and mucosa-associated invariant T (MAIT) cells, which are αβ T cells restricted to MHC-related protein 1 (MR1) (Bendelac et al. 2001; Treiner et al. 2003).

In determining the relative 'importance' of the various functions of any cell population, there is a distinct potential for observer bias to influence decisions. One approach to avoiding this bias is to determine the relative abundance of different signaling and effector molecules on a per-cell basis, with the presumption that a higher level of expression of a particular protein indicates a greater level of the activity in which that protein is involved. This type of analysis is particularly complex in the case of cytotoxic T lymphocytes (CTL), which are known to express 6562 proteins (Hukelmann et al. 2016). Analysis of CTL proteins by mass spectrometry and the 'proteomic ruler' method (Wisniewski et al. 2014) allows a determination of total protein mass, the relative abundance of each protein, and quantification of protein copy number per cell. These studies indicate that 249 proteins constitute >75% of the total CTL mass. Common proteins include those involved in basic cell structure, survival and replication, including histones, cytoskeletal proteins, ribosomal proteins and proteins involved in translation. However, molecules involved in immunological effector mechanisms and metabolism are the most commonly expressed proteins in CTLs, which gives insight into the relative importance of these functions of the cells. Cytotoxic granzyme A, granzyme B and perforin are at the forefront in copy number, with each CTL containing over 1×10^7 of each molecules per cell. This explains the long-standing observation that CTLs are able to rapidly and repeatedly kill target cells without taking time to replenish their arsenal of killing molecules (Isaaz et al. 1995).

Metabolic pathway components are also among the top copy numbers in CTLs. It is known that the metabolism of T cells changes with activation and differentiation, and that the metabolic changes are necessary for effective function (Buck et al. 2015). Activated CTLS are highly glycolytic, and reduction in glucose availability results in reduced ability to produce granzymes A & B, perforin and interferon-γ (Cham et al. 2008). The above analysis indicates that CTLs contain approximately 1×10^7 copies of multiple glycolytic enzymes per cell. This abundance of glycolytic enzymes allows CTLs to continue their function for a prolonged period, and perhaps have sufficient glycolytic reserves to participate in secondary 'moonlighting' activities such as RNA binding (Castello et al. 2015).

The studies of relative protein abundance in CTLs revealed substantial differences between proteomic data and transcriptomic data. For example, transcripts for IL-2Rα are present in CTLs in approximately twofold abundance over those for IL-2Rγ, but proteomic analysis indicates there are 50 copies of IL-2Rα for each molecule of IL-2Rγ (Hukelmann et al. 2016). This suggests that post-transcriptional pathways have a major role in shaping the proteome of CTLs (Sanin and Pearce 2015), and brings into further question the relationship between transcriptomics and proteomics.

Regulatory T cells (Tregs).

In addition to the major effector T cell populations, a population of regulatory T cells (Tregs) is involved in many (perhaps most) aspects of adaptive immunity. Tregs police the activity of other T cell populations, and serve to control or quench those effector T cell activities which are not beneficial to the host. The Treg population is divided into two major groups: central/natural Tregs and peripheral/inducible Tregs. Tregs control responses to self-antigens as well as inappropriate responses to food antigens and microbial populations in the intestinal microbiome.

Central/natural Tregs (cTregs) are thymic-derived, $Foxp3^+CD25^+CD4^+$ cells that constitute 5–10% of the total $CD4^+$ T cell population. They are characterized by the critical expression of Foxp3, a transcription factor that represses the expression of the T_H1, T_H2 or T_H17 cytokine profiles that characterize the effector T cell populations. Experimental evidence suggests that autoreactive T cells are present in normal individuals, and that the potentially harmful actions of these autoreactive T cells are controlled by Tregs. Elimination of the Treg function results in autoimmune disease such as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) in humans. See Volume 2, Chap. 13, Endocrine System for a further discussion of IPEX.

Unlike effector T cell populations, Tregs are incapable of producing the IL-2 that is crucial for their survival. The survival and function of Tregs depends on the paracrine production of IL-2 by other T cell populations. Reduction of IL-2 production by genetic modification or pharmacologic intervention would be expected to reduce the population and function of Tregs, and could be followed by autoimmune consequences.

Peripheral Tregs (pTregs) are derived from conventional $CD4^+$ T cells in the periphery, in contrast to the central/natural thymic Tregs that are derived from hematopoietic precursor cells in the thymus (Bilate and Lafaille 2012; Kretschmer et al. 2005; Mucida et al. 2005; Haribhai et al. 2011). Dietary antigens are rendered

nonimmunogenic by a process of "oral tolerance' that involves Foxp3$^+$ CD4$^+$ Treg cells, particularly pTregs (Weiner et al. 2011; Pabst and Mowat 2012). Using germ-free mice and antigen-free diets, it was demonstrated that the majority of small intestinal pTregs are induced by dietary antigens in solid food, while the majority of pTregs in the large intestine are induced by the local microbial flora. The food antigen-induced pTreg cells have a limited lifespan, and strongly repress immunity to dietary antigens. The food antigen-induced pTregs are distinguishable from pTreg cells that develop in response to microbial populations, as the food antigen-induced pTregs do not express the retinoic acid receptor-related orphan receptor γt (RORγt) that is expressed by microbially-induced pTregs (Lochner et al. 2008; Eberl 2012). These findings have relevance to observations that early postnatal exposure to peanuts serves to reduce the subsequent incidence of peanut allergies (Du Toit et al. 2015), and may have relevance to possible development of pharmacological interventions that would curb allergic responses to foods while preserving the immunologically necessary interactions with the intestinal microbiome.

γδ T cells.

Innate and adaptive immune responses have many of the same results with regard to interception of pathogens. The innate response has the advantage of immediate responsiveness, while the adaptive response has the advantage of immunological memory, which affords heightened and precisely focused responses to subsequent pathogen exposure. In the typical immunological paradigm, innate responses precede adaptive responses. However, there is reverse cross-talk by which adaptive immune cells contribute to the early recruitment and functional polarization of the innate immune cells (Bonneville et al. 2010). Conventional αβ T cell populations that participate in this reverse cross-talk include cells that are restricted to the non-polymorphic MHC class-1-like molecules CD1d and MHC-related protein (MR1, which are known as invariant NKT (iNKT) cells and mucosa-associated invariant T (MAIT) cells, respectively. An additional population of T cells, the γδ T cells, also participate in the reverse cross-talk by recognition of conserved non-peptide antigens that are up-regulated by stressed cells. The γδ T cells acquire a pre-activated phenotype early in their development, thus are able to mount a prompt response along with the response of innate immune cells. The γδ T cells are commonly located in epithelial surfaces that come into direct contact with the external environment. Expression of different TCRs by anatomically distinct populations of γδ T cells allows the subpopulations of γδ T cells to develop immunological responsiveness to the pathogens that prodominate in that anatomic site. Infected, activated or transformed cells are killed by pathways involving Fas and TNF-related apoptosis-inducing ligand receptors (TRAILR), and release of effector molecules such as perform and granzymes (Strid et al. 2008; Dieli et al. 2001). The γδ T cells also release a spectrum of signaling and effector molecules, including granulysin and defensins (Dieli et al. 2001; Dudal et al. 2006), TNF, IFNγ, IL-17, IL-4, IL-5, IL-13, IL-10 and TGFβ ((Bonneville et al. 2010). Some of these molecules mediate allergy and autoimmunity. A number of pharmaceutical products have been devised to capitalize on the antimicrobial and antitumor activities of γδ T cells (Bonneville and Scotet 2006; Sicard et al. 2005; Huang et al. 2009; Wilhelm et al. 2003; Dieli et al. 2007). See (Bonneville et al. 2010) for a review of γδ T cell effector functions.

2.2.2.2 B Lymphocytes and Plasma Cells

B cells develop in the bone marrow from a lineage that splits from the committed lymphoid progenitor (CLP) population (Hardy 2013). After progressing through a series of maturational steps that include rearrangements of the immunoglobulin heavy and light chains, naïve B cells relocate to the red pulp of the spleen. In the mouse it is estimated that 1–2 × 10^6 B cells are produced in the bone marrow each day, but only 10% of this number reach the periphery (Allman et al. 1993). Further maturation takes place in the splenic red pulp, during which the maturing cells are known as 'transitional B cells'. After completion of the maturational stages in the splenic red pulp, the B cells move into splenic follicles to constitute the recirculating pool of mature B cells. Follicular B cells in the mouse have a half-life of approximately 4.5 months (Hao and Rajewsky 2001).

It should be noted this B cell development pathway, which was derived in mice and subsequently found to be largely applicable to humans, may not be representative of B cell development in other species. B cell development in chickens, rabbits, sheep, swine and cattle has initial development of a limited BCR repertoire during fetal or early neonatal life, diversification of the BCR repertoire at a later time, and maintenance of the B cell pool during adult life via self-renewal rather than *de novo* generation from uncommitted bone marrow precursor cells.

Certain antigens, classified as thymus-independent antigens, are able to activate B cells without input from helper T cells. Bacterial lipopolysaccharides (LPS), if present in sufficient quantity, are able to activate a portion of the B cell population by binding to a component of the surface Ig other than the antigen-specific hypervariable region, plus additional surface receptors such as TLRs. At lower concentrations LPS is recognized by the specific antigen receptors on B cells, thus activating the cells by the traditional pathway. A second group of thymus-independent antigens have appropriately spaced repeating determinants that bind to multiple surface immunoglobulin molecules on B cells. These thymus-independent antigens remain in prolonged contact with the B cells, and send repeating first signals for activation. This group includes the polysaccharide coating of *Streptococcus pneumoniae* bacteria, $_D$-amino acid polymers, Ficoll and polyvinylpyrrolidone.

Antibody production, which is the major effector function of B cells, is dependent on the generation and maintenance of plasmablasts and plasma cells. Plasmablasts are antibody-secreting cells of the B cell lineage that retain ability to divide and migrate. Some plasmablasts mature into plasma cells, which are terminally differentiated, non-replicating cells that are characterized by their ability to secrete large amounts of antibody. Mixed populations of plasmablasts and plasma cells are known collectively as *antibody secreting cells* (ASCs)(Fairfax et al. 2008).

In T-dependent immune responses, antigen-activated proliferating B cells can take one of three pathways to become (a) short-lived extrafollicular plasma cells, (b) germinal center-dependent memory cells, or (c) germinal center-independent memory cells. This commitment, which apparently occurs before the proliferating B cells enter into the germinal center cycle, involves isotype switching but cannot involve the somatic hypermutation that is restricted to germinal centers. B cells

expressing antibodies of the highest affinity enter into longer term contact with T_{FH} cells at the B cell-T cell junction in the spleen or lymph nodes, therefore receive a greater level of T_{FH} help and are more likely to enter the germinal center cycle (Schwickert et al. 2011). If the period of contact between B cells and T_{FH} cells is relatively short, then the B cells are more likely to become germinal center-independent memory B cells. Thus the preference for B cells expressing high-affinity surface antibody is in place even before cells enter the germinal center cycle, which enhances and refines the selection process (Allen et al. 2007).

The generation of ASCs in response to thymus-dependent (TD) antigens is a two-step process (Nutt et al. 2015). The first step (the 'interfollicular response') involves activation of B cells by specific antigen, followed by B cell replication to form short-lived plasmablasts that secrete the moderate-affinity antibodies of the early immune response (MacLennan et al. 2003). The intrafollicular response can involve antibody class-switching, but exhibits little of the somatic hypermutation that generates high-affinity antibodies. In the second step of the TD response, some of the activated B cells enter the B cell follicle, where they interact with T follicular helper (T_{FH}) cells and then actively proliferate in a germinal center (Victora and Nussenzweig 2012; Shlomchik and Weisel 2012; Nutt and Tarlinton 2011). The germinal center reaction produces long-lived plasma cells that produce high-affinity antibodies (Shlomchik and Weisel 2012; Radbruch et al. 2006). The germinal center reaction also produces memory B cells that rapidly differentiate into active ASCs following re-exposure to antigen (Kometani et al. 2013). The two-step process provides for an immediate antibody response to antigenic challenge, a slightly delayed high-affinity antibody response, and a cadre of memory B cells that are programmed to rapidly respond to a second pathogen challenge. A microRNA (miR-155) has been shown to regulate cytokine production that is critical for the generation of germinal centers (Thai et al. 2007).

Long-lived plasma cells exist primarily in the bone marrow, though a few long-lived plasma cells are known to exist in other lymphoid and non-lymphoid organs (Smith et al. 1997; Benner et al. 1981; Slifka et al. 1995). The marrow plasma cell population exists in a finite number of plasma cell 'niches' which consist of a collaboration between $CSCL12^{+}$ $VCAM1^{+}$ stromal cells and eosinophils (Chu et al. 2011) or other hematopoietic cells (Winter et al. 2011) that produce the B cell survival factor known as 'a proliferation-inducing ligand' (APRIL) (Nutt et al. 2015). The bone marrow environment is necessary for maintenance of the long-lived plasma cell population, as displacement of the plasma cells from the marrow microenvironment results in rapid death of the long-lived plasma cells (Chu and Berek 2013; Yoshida et al. 2010). Toxicologists and toxicologic pathologists should be particularly concerned at the potential long-term effects of the transient bone marrow depletion that commonly occurs following administration of some classes of test articles, e.g., chemotherapeutic agents, as the transient decrement in hematopoietic elements may result in an invisible effect on the long-lived plasma cell population and subsequent host susceptibility to pathogens.

The immune system can remember a previously experienced pathogen and mount an enhanced response upon a second exposure to that pathogen. There are

two components to this long-term humoral defense against pathogens. The first component, constitutive humoral immunity, is long-term production of specific antibodies by bone marrow plasma cells. Constitutive humoral immunity is adequate only if there is a sufficient level of specific antibody present at the site of reinfection. If the immediately available constitutive humoral immunity proves to be inadequate, a second line of defense (reactive humoral immunity) in the form of memory B cells is activated. The reactive humoral response, while not immediate, typically develops faster than the original primary immune response. It also is typically of greater magnitude and consists of high-affinity antibodies with switched isotypes (Ahmed and Gray 1996). Many of the memory B cells that participate in the reactive humoral response evolve from high-affinity, IgG^+ B cells that are generated in the germinal center reaction (Berek et al. 1991; Liu et al. 1996). However, there is evidence for germinal center independent memory B cells (Anderson et al. 2007; Taylor et al. 2012; Kaji et al. 2012), as well as memory B cells that have not switched from IgM to IgG production (Klein et al. 1997; Dogan et al. 2009; Pape et al. 2011).

See (Nutt et al. 2015) for a review of plasma cell biology.

B1 cells were initially described as B cells that express CD5, which is largely a T cell marker, but later information has revealed cells with the B-1 phenotype that are $CD5^-$. B1 cells are very common during ontogeny and in young neonatal animals, but in mature animals the B1 cell population is diminished. B1 cells in adult animals are found primarily in the peritoneal and pleural cavities, where they constitute 10–30% of the B cell population. A substantial population of B1 cells is also present in the spleen, but is numerically overwhelmed by the large population of traditional B cells. The B1 cell population is maintained by self-renewal, as opposed to reconstitution from bone marrow precursors (Hayakawa et al. 1986). It should be noted that most rabbit B cells express CD5 (Raman and Knight 1992).

B-1 cells also have a distinctive characteristic of producing auto-antibodies to specific molecules such as branched carbohydrates, glycolipids, glyproteins, and bacterial cell wall and capsule constituents (Hardy 2013). These autoantibodies, referred to as ‘natural autoantibodies’ are not pathogenic. They are thought to provide for elimination of effete cells or proteins, or provide immediate defense against certain pathogens. The latter ability represents a form of genetically predetermined immunologic memory, possibly a result of evolutionary exposure to these pathogens.

Marginal zone B (MZB) cells are concentrated in the marginal zone of the spleen, which is an area of maximal antigen exposure to a co-located subpopulation of macrophages (‘marginal zone macrophages’). MZB cells have a BCR repertoire similar to that of B-1 cells, and are preselected to recognize bacterial wall components (Shaw et al. 2000) and components of senescent cells (Silverman et al. 2000; Shaw et al. 2000). Among all B cell populations, MZB cells are unique in depending on Notch 2 signalling for their development (Saito et al. 2003; Kuroda et al. 2003). MZB cells mount an early response to antigenic challenge, and are able to respond to bacterial pathogens with thick polysaccharide coatings that may present a challenge to the traditional thymus-dependent immune response. One such bacterial pathogen is

Streptococcus pneumoniae, the cause of potentially fatal lobar pneumonia in humans. Loss of the MZB population following splenectomy substantially raises the risk for developing lobal pneumonia, therefore immunization against that pathogen is commonly administered following splenectomy (Theilacker et al. 2016; Shaw and Print 1989; Waghorn 2001). A number of immunomodulatory drug candidates cause pronounced reduction in marginal zone populations in the spleen, and may increase the potential for complicating bacterial infections.

2.2.2.3 Dendritic Cells

Dendritic cell immunobiology is a particularly complex topic, thus the following is only a superficial overview. Dendritic cells (DCs) provide a conduit between the innate and adaptive immune systems (Liu and Nussenzweig 2013). DCs in the periphery serve a sentinel function, whereby their surface expression of pattern recognition receptors (PRRs) allows them to detect pathogen and danger signals. Macrophages have some of the same functions but, in contrast to macrophages, DCs do not engage in active immunologic resistance at the site of infection or inflammation. Instead, DCs migrate through lymphatics to local lymph nodes, where they activate a population of lymphocytes that assume the effector functions in resistance. DCs incorporate antigenic peptides into MHC molecules for presentation to T cells as the first signal in activation. DCs also express CD80/86 (previously known as B7-1 and B7-2) which serve as co-stimulatory (second) signals that bind to CD28 on T cells. The expression of co-stimulatory CD80/86 by DCs occurs only after DCs contact PAMPs or DAMPs (pathogen-associated molecular pattern or danger-associated molecular pattern, respectively). CD80/86 expression by DCs is also regulated by the NFκB signaling pathway, which is the common downstream pathway associated with many PRRs. If DCs present antigenic peptides in the absence of co-stimulatory CD80/86, it is most likely because the peptide was derived from a self-protein that was not recognized as a PAMP or DAMP signal, therefore the DCs are not activated. T cells that interact with these antigenic peptides in the absence of the co-stimulatory signal from activated DCs become tolerized rather than immunologically activated. The peripheral tolerance can be intrinsic via T cell deletion or anergy, or extrinsic via peripheral generation of Treg cells.

The thymic population of cDCs, along with the thymic epithelial cell population, is essential in the negative selection process that eliminates self-reactive thymocytes. Thymic epithelial cells, under the control of the autoimmune regulator AIRE (Anderson 2002), express numerous self-antigens. Thymic cDCs also capture and present self-antigens. This combined presentation of self-antigens is the basis for the negative selection process. Thymic cDCs also promote the development of Treg cells in the thymus, thus contributing to thymic-origin central tolerance (Proietto et al. 2008).

DCs process ingested antigen for presentation by both MHC class I and class II. The MHC class II antigen presentation by DCs is different from that of macrophages, in which ingested antigen is rapidly digested. DCs assimilate antigen and

preserve it for long-term presentation, largely due to the low levels of proteases and the less acidic environment of DC lysosomes compared to macrophage lysosomes (Delamarre et al. 2005; Trombetta et al. 2003). These features of DC lysosomes reduce the rate of protein degradation and increase the period of time during which polypeptides are available for MHC presentation. The activation of DCs includes redistribution of MHC class II molecules from intracellular storage compartments to the plasma membrane. DCs present endogenous antigens in MHC class I to $CD8^+$ T cells via the classic endogenous antigen presentation pathway. In an unusual development, it has been found that DCs can also present **exogenous** antigens in context of MHC class I. This is accomplished by a process known as 'cross-presentation' in which DCs take up external antigens such as viruses or fragments of apoptotic cells and present them to $CD8^+$ T cells in context of MHC class I (Jung et al. 2002), thus promoting the development of CTLs that attack tumor cells or virus-infected cells. This phenomenon of cross-presentation is critical for defense against viruses and tumors.

DCs have numerous interactions with cells of the adaptive immune system. DCs interact with innate lymphocytes (NK, NKT and γδ T cells) in ways that facilitate the activities of both cells. E.g., DCs produce cytokines that affect NK cell function, and the innate lymphocytes activate DCs (Munz et al. 2005; Walzer et al. 2005). DCs are involved with T cell fate decisions such as clonal selection, tolerance versus immune activation, T_H1 versus T_H2 diversity, and T cell memory.

In the mouse the DC population is divided into three major subsets: conventional DCs (cDCs), plasmacytoid DCs (pDCs) and migratory DCs (mDCs). The cDC population is further subdivided into $CD8\alpha^+$ and $CD8\alpha^-$ subgroups. The $CD8\alpha^+$ subgroup is biased toward MHC class I antigen presentation, while the $CD8\alpha^-$ subgroup is biased toward MHC class II antigen presentation (Dudziak et al. 2007). The pDC subset is highly involved in antiviral immunity due to their production of type 1 interferons. The mDC subset, which is subdivided into $CD103^+$ and $CD103^-$ subgroups (Helft et al. 2010), is named because the DCs exist in peripheral tissues and migrate to lymph nodes upon recognition of pathogen or danger signals (Shortman and Liu 2002; Banchereau and Steinman 1998).

Dendritic cells develop from hematopoietic stem cells in the bone marrow in conjunction with leukocyte populations. At an early stage in development, the emerging leukocyte population is split into myeloid progenitors (CMP-common myeloid progenitor) that eventually form monocytes, macrophages, granulocytes, megakaryocytes and erythrocytes, and lymphoid progenitors (CLP-common lymphoid progenitors) that eventually form lymphocytes and NK cells. Though it was originally thought that DCs develop from both CMP and CLP populations, current information suggests that DCs develop exclusively from the CMP cells (Manz et al. 2001). The developmental sequence includes MDPs (macrophage-dendritic cell porgenitors), which eventually produce monocytes, macrophages and dendritic cells, and then CDPs (committed dendritic cell progenitors), which produce only dendritic cells (Liu et al. 2009).

DCs have previously been considered a part of the monocyte/macrophage group of cells, and there is substantial literature regarding MODC (monocyte-origin DCs).

Monocytes can develop some features of DCs under inflammatory conditions or when exposed to cytokines *in vitro*, but current considerations indicate DCs are not derived from monocytes (Liu and Nussenzweig 2013). There is debate as to whether DCs represent a *de novo* cell population that is derived directly from the CMP cells, or represent a modification of the macrophage cell line. Arguments in favor of the latter proposal are eloquently presented by (Hume 2008) as the 'dendritic cell myth'.

'Interdigitating cells' located in the T cell zones of the spleen and lymph nodes were initially thought to be macrophages, but further study has revealed them to be cDCs. The cDC population in secondary lymphoid organs is largely non-motile, but they continually extrude and contract cytoplasmic processes into their local environment (Lindquist et al. 2004). (These processes resemble the dendrites of neurons, thus the name 'dendritic cells'). The cDC population forms a network within the T cell-dependent areas of secondary lymphoid organs, and incoming T cells migrate through the cDC maze in search of the specific cognate antigen that the T cells is programmed to recognize. When a T cell encounters a cDC expressing the cognate antigen, there is a prolonged interaction (approximately 18 h) in which the T cell becomes activated and subsequently proliferates to form a clone of antigen-specific T cells.

Plasmacytoid dendritic cells (pDCs) are a subset of DCs that specialize in the production of type I interferons (IFNs), thus are important in responses to viral infection (Siegal et al. 1999; Cella et al. 1999) and autoimmune diseases that are characterized by IFN type I (Ganguly et al. 2013). Production of type 1 IFNs is stimulated by the recognition of viruses or self nucleic acid by TLR7 and TLR9 on pDCs (Gilliet et al. 2008; Blasius and Beutler 2010; Kawai and Akira 2011). DCs and pDCs are derived from a common bone marrow progenitor cell that lacks lineage markers for other hematopoietic cell lines (Naik et al. 2007; Onai et al. 2007; Reizis 2010; Satpathy et al. 2012). The migratory pattern of pDCs is different from that of classical DCs. Following development in the bone marrow, pDCs circulate in the blood as 'veiled cells' (Petrella and Facchetti 2010), and reach T cell-dependent areas of lymph nodes via high-endothelial venules (HEV), as opposed to the cDC pathway of entering lymph nodes via afferent lymphatics (Penna et al. 2001; Sozzani et al. 2010). pDCs also migrate into non-lymphoid peripheral tissues, and are selectively recruited into areas of inflammation (Krug et al. 2002; Diacovo et al. 2005). pDCs migrate in response to engagement of receptors for chemerin and adenosine, thus are actively recruited into sites of tissue damage (Sozzani et al. 2010). Recruitment of pDCs into neoplasms is a negative prognostic indicator, as pDCs tend to induce tolerance rather than immunity (Munn et al. 2004; Treilleux et al. 2004; Sisirak et al. 2012).

Plasmacytoid DCs express MHC class II as well as the co-stimulatory molecules CD40, CD80 and CD86, thus they can present antigens to $CD4^+$ T cells without additional help (Reizis et al. 2011; Villadangos and Young 2008).

It is known that mDCs are found in most organs of the body, but they are concentrated in organs that have direct contact with the environment. They are most commonly found in interstitial spaces, from which they exit via the lymphatics (Hart and

Fabre 1981; Anandasabapathy et al. 2011; Ginhoux et al. 2009). Migratory DCs in peripheral organs constantly sample potentially antigenic peptides in their environment, then travel in lymphatics to local lymph nodes where the peptides are presented to T cells. While in the lymphatics the mDCs have long cytoplasmic processes, resulting their designation as 'veiled cells' (Knight et al. 1982; Kelly et al. 1978; Drexhage et al. 1979; Bujdoso et al. 1989). While enroute in the lymphatics the mDCs undergo maturation that increases their efficiency as antigen-presenting cells. DCs are not present in efferent lymph from lymph nodes, suggesting the DCs perish in the lymph nodes as opposed to recycling out to peripheral tissues.

Further study has revealed that mDCs in different organs have developed different capabilities, presumably in response to the types of threats that are prevalent in those organs. Subsets of DCs are known in some organs. For example, the mDCs of the epidermis, traditionally known as Langerhans cells, are phenotypically and functionally different from mDCs located in the dermis. In addition, the origin of Langerhans cells (LCs) of the epidermis is different from that of cDCs. LCs are derived from yolk sac or fetal liver progenitor cells (Schulz et al. 2012), and consist of a long-lived cell population that replicates in the skin (Merad et al. 2002; Merad et al. 2004). Bone marrow-origin cDCs are recruited to the skin only in association with inflammatory reactions. By contrast, the cDC population in the dermis is similar to cDC populations in other organs, and is continually replenished by immature DCs from the bone marrow. The LC population in the skin is reminiscent of the tissue resident macrophage population in multiple organs, as presented under Macrophages.

Follicular dendritic cells (FDCs) are a unique population of cells that are essential for germinal center formation and production of high-affinity antibodies in secondary lymphoid organs (Tew et al. 1990). They develop from stromal origin precursors which are seeded throughout the body, with some coming to rest in a central location in germinal center (Alimzhanov et al. 1997; Endres et al. 1999; Pasparakis et al. 1996). FDCs produce CXC-chemokine ligand 13 (CXCL13), which signals through CXCR5 to attract B cells and specific subsets of T cells to the follicles (Ansel et al. 2000). They promote B cell survival in germinal centers through the production of IL-6 and B cell activating factor (BAFF)(Garin et al. 2010; Wu et al. 2009). FDCs have the unique ability to retain intact antigen for a prolonged period, which is required for germinal center maintenance, B cell somatic hypermutation that results in high-affinity antibodies, and promotion of long-term memory (Kelsoe 1996). The germinal center reaction starts with migration of activated B cells to the T cell/B cell junction of the primary lymphoid follicle, where the activated B cells present antigen to helper T cells and receive co-stimulation. Following this initial interaction, some of the B cells migrate to the medullary cords of the lymph node, where they produce low-affinity antibodies. However, a selected population of B cells enters into repeated cycles of the 'germinal center reaction', during which sequential steps of somatic hypermutation, selection and cellular proliferation result in a population of B cells that produce high-affinity antibodies. This population of high-affinity B cells generates a population of high-affinity plasma

cells that migrate to the bone marrow, where they produce high-affinity antibodies for a prolonged period.

FDCs arise from vascular mural cells that are seeded throughout the body (Castagnaro et al. 2013; Krautler et al. 2012), and occasionally give rise to FDC sarcoma (Chan et al. 1997). FDCs have some phenotypic similarities to fibroblastic reticular cells (FRC) and marginal reticular cells (MRC). FDCs are not phagocytic, and do not have a shared origin with bone marrow-origin cDCs. FDCs of mice are the only cells known to retain opsinized antigen for long periods of time (Kelsoe 1996), possibly for years after antigen exposure (Tew and Mandel 1979). Lymph bearing soluble or particulate antigen enters lymph nodes via the subcapsular sinuses, where the antigens encounter conduits formed by FRC and interact with FDCs in B cell follicles. The soluble antigens proceed directly to their destination, while particulate antigens are phagocytized by subcapsular sinus macrophages that escort the antigens to their final destination (Phan et al. 2007; Junt et al. 2007; Carrasco and Batista 2007). Immune complexs that arrive in the afferent lymph are taken up by subcapsular sinus macrophages via Fc receptors and complement receptor 3. Aggregations of antibodies, antigens and complement appear as the 'iccosomes' that are visible on the surface of FDCs by electron microscopy. Some of the CR2-bound C3d-coated immune complexes are internalized into non-degradative endosome compartments (Heesters et al. 2013), where they are available for long-term stimulation of B cells. In this way the immune system can capture some of the antibody produced in the initial immune response, use that antibody to capture antigen, and store the antigen/antibody/complement complex for prolonged periods. This process does not constitute 'immunologic memory' as it is currently defined, but it provides a mechanism whereby an antibody response can be prolonged or reconstituted after a prolonged interval.

In addition to various changes in immunocyte phenotype and mediator expression, the germinal center reaction also involves a carefully orchestrated movement of B cells within the germinal center. Somatic hypermutation takes place in one region of the germinal center, while cellular proliferation takes place in another region, and cells must physically relocate to undergo the various steps. Descriptions of these processes commonly refer to 'light zones' and 'dark zones' in germinal centers, but it should be noted these zones appear only in secondary lymphoid organs of humans and, less distinctly, nonhuman primates. The light and dark zones are not readily apparent in germinal centers of rodents and dogs.

See (Heesters et al. 2014) for a review of follicular dendritic cells. See (Fletcher et al. 2015) and (Link et al. 2007) for a review of fibroblastic reticular cells.

DC-SIGN (DC-**Sp**ecific **I**ntercellular adhesion molecule-**G**rabbing **N**onintegrin) has such an important role in DC immunobiology that a specific presentation is warranted.

Immature DCs located throughout the body act as immunological sensors that detect pathogens largely via recognition of pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) and C-lectins on the DCs. Once a DC has recognized a pathogen, it migrates to a regional lymph node to present representative antigenic peptides to T cells. During the process of migration the DC undergoes

maturational changes that include expression of co-stimulatory molecules, thus making the DC capable of providing both the first (antigenic peptide in MHC context) and second (co-stimulatory molecule expression) signals that are necessary for activation of naïve T cells (Mellman et al. 1998). Depending on the characteristics of the pathogen recognized by the DCs, the naïve T cells differentiate into T_H1 cells that produce IFN-γ or T_H2 cells that produce IL-4 (de Jong et al. 2002). For example, the yeast form of *Candida albicans* induces IL-12 production by DCs, which results in T_H1 differentiation of naïve T cells, while the hyphal form of *Candida albicans* inhibits IL-12 production and promotes IL-4 production, resulting in T_H2 differentiation of naïve T cells (d'Ostiani et al. 2000). This induction of T_H1 versus T_H2 responses is manipulated by some pathogens to promote their survival.

Many different C-type lectins exist on the surface of DCs, and many of these lectins function as antigen receptors for carbohydrate components in the cell walls of pathogens (Figdor et al. 2002). Recognition of the pathogen components by DC lectins allows internalization of pathogens followed by lysosomal degradation and presentation of pathogen-associated antigenic peptides by MHC molecules on the surface of DCs (Figdor et al. 2002; Engering et al. 2002). The DC surface lectins include the mannose receptor (CD206)(Sallusto et al. 1995), DEC205 (CD205) (Mahnke et al. 2000), DC-SIGN (CD209) (Geijtenbeek et al. 2000), blood DC antigen 2 (BDCA2) (Dzionek et al. 2001), dectin-1 (Willment et al. 2001), DC immunoreceptor (DCIR) (Bates et al. 1999), DC-associated lectin 1 (DCAL1) (Ryan et al. 2002), C-type lectin receptor 1 (CLEC1) (Colonna et al. 2000), Langerhans-cell-specific C-type lectin ((Langerin), (Valladeau et al. 2000), DC-asialoglycoprotein receptor (DC-ASGPR) (Valladeau et al. 2001), and macrophage galactose *N*-acetyl-galactosamine specific lectin 1 (MGL1) (Suzuki et al. 1996). C-type lectins are expressed most commonly by DCs in skin or mucosal tissues, with lower C-type lectin expression by Langerhans cells or DCs in the blood (van Kooyk 2003).

DC-SIGN is expressed by dermal DCs and interstitial DCs in the lungs, intestine, cervix, placenta and lymph nodes. DC-SIGN recognizes ICAM2 and ICAM3, and functions as a cell-adhesion receptor that regulates DC migration (van Kooyk and Geijtenbeek 2002) and DC-T cell interactions (Geijtenbeek et al. 2000). DC-SIGN-specific antibodies inhibit DC-T cell clustering and DC-induced proliferation of T cells (Geijtenbeek et al. 2000), thus demonstrating the central role of DC-SIGN in DC-T cell interactions. DC-SIGN binding to ICAM2 on endothelial cells functions as a rolling receptor that allows DC precursors to migrate from blood to tissues (van Kooyk and Geijtenbeek 2002). This latter function of DC-SIGN is similar to the function of selectin molecules, which also are C-type lectins.

In addition to having a general function in DC-T cell interactions, DC-SIGN acts as a receptor for viral envelop glycoproteins of a number of important viral pathogens, including HIV-1, HIV-2, simian immunodeficiency virus, Ebola virus, cytomegalovirus, hepatitis C virus and Dengue virus (van Kooyk 2003). Differential glycosylation of the viral envelope glycoproteins of some of these viruses affects the binding of DC-SIGN and affects the ability of the viruses to infect target cells (Lue et al. 2002). DC-SIGN on immature DCs captures HIV-1 virus in peripheral tissues and transports the virus to lymph nodes, where transmission of the virus to

$CD4^+$ T cells occurs (Steinman 2000) (vK ref. (Cameron et al. 1992; Pope et al. 1994)). A number of non-viral pathogens such as *Helicobacter pylori*, *Klebsiella pneumoniae, Mycobacterium tuberculosis, Leishmania pifanoi, Schistosoma mansoni,* and *Candida albicans* are also recognized by DC-SIGN. The carbohydrate profiling of DC-SIGN determines which organisms are internalized, degraded, and eventually have their antigenic peptides presented to T cells by the DCs. A common feature of this group of pathogens is their tendency to cause chronic, 'smoldering' diseases that in many ways represent a stalemate between the pathogens and the immune system. Manipulation of the T_H1–T_H2 balance by the pathogens is central to their persistence. This is demonstrated by mycobacterial targeting of DC-SIGN to suppress DC function and modulate immune responses by shifting the T_H1 versus T_H2 balance (Geijtenbeek et al. 2003).

The central role of DC-SIGN in DC function and demonstrated involvement of DC-SIGN in the immunomodulation associated with multiple pathogens suggests DC-SIGN biology could be an attractive pharmacologic target for interception of a number of important human diseases. See (van Kooyk 2003) for a review of DC-SIGN immunobiology.

2.2.2.4 Stromal Cells

Stromal cells were once defined as non-hematopoietic cells that were adherent in cell culture (Friedenstein et al. 1974), but the term is now applied to non-hematopoietic cells that form a matrix (Tokoyoda et al. 2010). Stromal cells in secondary lymphoid organs (SLOs) are classified as fibroblastic reticular cells, follicular dendritic cells, marginal reticular cells, red pulp fibroblasts (spleen), medullary fibroblasts (lymph nodes), integrin α7 pericytes (Chang and Turley 2015), lymphatic endothelial cells, and vascular endothelial cells (Mueller and Germain 2009). An additional population of stromal cells, including mesenchymal stromal cells, endothelial cells, osteoblasts, and adipocytes, serve multiple functions in the bone marrow.

Lymphoid tissue organizer (LTo) cells derived from adipocyte precursors cooperate with lymphoid tissue inducer (LTi) cells of hematopoietic origin to initiate the formation of lymph nodes, thus stromal cells are present in SLOs from the onset. The stromal cells direct, nourish, and instruct immunologically active cells throughout life. Stromal cells of the secondary lymphoid organs (SLOs) contribute to immune responses by acting as scaffolds for cell trafficking, providing cytokines and chemokines, presenting antigen, and expressing adhesion and/or inhibitory molecules (Mueller and Germain 2009).

The function of the stromal cells depends as much on their physical location as their phenotype. Stromal cells of lymph nodes are positioned to guide incoming dendritic cells and soluble antigens from the entry point in the subcapsular sinuses to areas of potential contact with helper T cells and primary B cell follicles. Lymphocytes arrive in lymph nodes primarily via high-endothelial venules (HEVs), which are concentrated in regions known as 'cortical ridges' located between the B

and T cell zones (Katakai et al. 2004b; Katakai et al. 2004a). T cell zones of lymph nodes contain $CD4^+$ and $CD8^+$ T cells along with subsets of dendritic cells, all anchored to a network of fibroblastic reticular cells and reticular fibers. B cell follicles contain a network of follicular dendritic cells and a peripheral network of stromal cells that border the T cell zones, and serve to facilitate contact between naïve B cells and T cells. The peripheral network of stromal cells is phenotypically and functionally similar to the marginal reticular cells of the spleen (Katakai et al. 2008).

An enormous repertoire of antigen receptors exists in the immune system to match the vast array of potential antigens that may be encountered in a lifetime. Because of the diversity of the repertoire, it is not possible to have more than a very small number of naïve T cells bearing each specific antigen receptor that must contact an antigen-bearing dendritic cell. If these interactions occurred in a distributive fashion, where the reactants randomly encounter each other, there would be little chance for the specific antigen receptor to make contact with the cognate antigen on a dendritic cell. However, observations indicate the immune system is able to initiate immune reactions with remarkable speed and reliability. This suggests the interactions occur in a processive fashion, i.e., the reactants are guided into contact by some external influence. Much of this guidance is provided by various stromal components of the immune system.

Lymphocytes bearing receptors for specific antigen continuously search through lymphoid tissues in a never-ending quest for cognate antigen. Lymphocytes enter lymph nodes via high-endothelial venules (HEVs), percolate through the sinuses and channels of the lymph node, and exit the lymph node into efferent lymphatics that eventually flow into the major lymphatic vessel known as the thoracic duct. The thoracic duct empties into the general venous circulation, thus returning the lymphocytes to their starting point to repeat the cycle. The lymphocyte population of the lymph nodes and spleen remains relatively constant despite the continual movement of lymphocytes, suggesting ingress and egress are somehow equalized (Chang and Turley 2015). While this ingress/egress balance is not fully clarified, there is evidence that HEVs serve as a temporary holding pen for incoming lymphocytes (Mionnet et al. 2011), which are released into the lymph node parenchyma when the resident lymphocyte population declines. The distinctive cuboidal endothelium of HEVs results from nesting of lymphocytes in pockets on the abluminal side of the endothelial cells, which constitute a way-station for migrating lymphocytes as they exit the circulation and rest for some period of time before being released into the lymph node. Incoming lymphocytes are not released from the HEV holding pen until departure of lymphocytes in sinuses of the lymph node make space available for additional incoming lymphocytes. In this way the HEVs serve as a ‘throttle’ that limits the ingress of lymphocytes.

Lymphocyte egress from lymph nodes begins at the blunt-ended cortical sinuses that populate the paracortex, and proceeds via the medullary sinuses to the efferent lymphatics (Grigorova et al. 2010; Grigorova et al. 2009). Lymphocyte egress is dependent largely on lymphocyte expression of sphingosine-1-phosphate receptor (1S1PR1) and a differential concentration of S1P within lymph and lymph node

tissue (Matloubian 2004; Schwab et al. 2005; Pham et al. 2008). S1P is largely absent within the lymph node parenchyma, but a high concentration is maintained in blood and lymph through contributions by hematopoietic cells and lymphatic endothelial cells (Pappu et al. 2007; Pham et al. 2010). Naïve lymphocytes entering the lymph node are exposed to S1P, which binds to the S1PR1 receptor. The SIP/S1PR1 complex is rapidly internalized into the lymphocyte, and S1PR1 receptor does not re-appear on the surface of the lymphocyte until additional molecules are synthesized (Grigorova et al. 2010; Lo et al. 2005). Absence of S1PR1 on the surface of lymphocytes that recently arrived via the HEVs prevents the egress of those lymphocytes via the sinuses that are commonly in close proximity to HEVs. This explains the lack of lymphocyte egress immediately after entry into lymph nodes, but does not explain the normal residency period of lymphocytes in lymph nodes (6–10 h for T cells and 12–24 h for B cells). More recent information suggests production of IL-7 by fibroblastic reticular cells has a major influence on duration of lymphocyte occupancy in lymph nodes.

During periods of immunologic stimulation, lymphocyte recruitment into lymph nodes is enhanced and egress is transiently diminished (Hall and Morris 1965). The enhanced recruitment is accomplished largely through the action of cytokines and chemokines elaborated by the inflammed peripheral tissue, which move through the lymph node cortex via conduits formed by fibroblastic reticular cells that extend from the subcapsular sinuses to the HEV's (Baekkevold et al. 2001; Palframan et al. 2001; Sixt et al. 2005). These factors transcytose across the HEVs and are displayed on the luminal surfaces of the HEVs, where they can attract blood-borne naïve circulating lymphocytes (Baekkevold et al. 2001). Signals from the inflammed tissue also cause up-regulation of CD69 on lymphocytes, which results in reduced responsiveness to S1P and a transient halt to lymphocyte egress from lymph nodes (Shiow et al. 2006).

Lymph nodes undergo major structural alterations in response to immunologic challenge. Changes to the primary arteriolar structure brings a greater supply of blood to the lymph node (Soderberg et al. 2005). HEVs grow in size and number in proportion to the increased size of the lymph node, thus the proportional density of HEVs remains constant (Kumar et al. 2012; Webster et al. 2006). Dendritic cells influence the vascular expansion by enhancing the production of VEGF by FRCs (Chyou et al. 2008). The FRC network also undergoes proliferative expansion to accommodate the increased number of lymphocytes in the lymph node (Chyou et al. 2011; Yang et al. 2014).

Though the capture and transport of antigens to regional lymph nodes by DCs is a major component of the immune response, a substantial number of antigens are delivered to lymph nodes as soluble molecules contained within lymph. Lymph originates as interstitial fluid, which enters the blind-ended lymphatic capillaries that are distributed throughout tissues (Baluk et al. 2007). The loosely arranged endothelial cells lining the lymphatic capillaries have 'button-like' intercellular junctions that insure unidirectional flow of interstitial fluid into the capillary lumen. Down-stream elements of the lymphatic system have smooth muscle that pumps the lymph toward lymph nodes and luminal valves that ensure unidirectional flow

toward the lymph node (von der Weid 2001). Dendritic cells help direct chemotaxis by secreting CCL19, which diffuses freely and moves with the flow of interstitial fluid/lymph (de Paz et al. 2007; Randolph et al. 2005). Migration of DCs within lymphatic capillaries depends on DCs crawling along the endothelial surface, with directionality linked to the rate of fluid flow (Tal et al. 2011). Once they reach larger lymphatic vessels, DCs are passively swept along by the flow of lymph until they reach lymph nodes (Tal et al. 2011).

The lymph drainage from peripheral tissues transports soluble antigens to lymph nodes within minutes of release of the antigens from the peripheral tissues. Small antigenic molecules, i.e., less than approximately 70 kDa molecular weight, permeate lymph nodes via the conduits formed by fibroblastic reticular cells. This conduit system quickly brings small antigenic molecules into contact with resident DCs, follicular DCs and cognate B cells (Sixt et al. 2005; Roozendaal et al. 2009; Roozendaal et al. 2008). Larger antigenic particles such as viruses are captured by macrophages in the subcapsular and medullary sinuses (Nossal et al. 1968), which transmit the antigens to cognate B cells in the cortex of the lymph node (Carrasco and Batista 2007; Phan et al. 2007; Junt et al. 2007). Proliferating LECs of subcapsular sinuses, which develop in response to inflammation, also capture and store antigen (Tamburini et al. 2014). These systems result in the capture of the great majority of antigenic material that reaches the lymph node (Drinker et al. 1934), thus preventing the systemic dissemination of pathogens (Junt et al. 2007; Iannacone et al. 2010; Gonzalez et al. 2010).

Secondary lymphoid organs are organized into distinct compartments, and orderly movement between these compartments is essential for effective immune functioning. If these microanatomical relationships are disrupted, then major disruptions of immune cell movement and overall immunologic function may result. Lymphoid organs tend to have dense populations of lymphocytes, and cellular movement is dependent on close interactions between lymphocytes and stromal cells (Chang and Turley 2015; Bajenoff et al. 2006). Lymphocytes crawl along the meshwork of stromal cells, but are retained in certain geographic regions that are delineated by chemokine expression patterns (Bajenoff et al. 2006). In lymph nodes, T cell entry and retention in the paracortex is mediated by expression of CCR7 and its recognition by CCL19 and CCL21, and naïve B cells entering lymph nodes rely on CXCR5 interactions with stromal expression of CXCL13 to locate the cells within follicles (Forster et al. 1996; Gunn et al. 1998a, 1998b).

Recognition of the importance of stromal cell function in overall immunologic function has resulted in an explosion of information on the topic. Following are very brief summaries of the functions of various stromal cell populations.

Fibroblastic reticular cells (FRCs) produce the collagen fibers that constitute the supportive lattice of lymphoid organs (Katakai et al. 2004a), including lymph nodes, spleen, thymus and other lymphoid tissues. FRCs constitute 20–50% of the non-hematopoietic cell population of lymph nodes (Link et al. 2007; Fletcher et al. 2011), where they form a three-dimensional network that guides lymphocytes as they migrate through the lymph node. FRCs also produce a web of extracellular matrix components that form a conduit system which transports antigens and soluble

molecules deep into the lymph node (Roozendaal et al. 2009; Gretz et al. 2000; Malhotra et al. 2013). FRCs are immunologically specialized myofibroblasts of mesenchymal origin (Link et al. 2007; Katakai et al. 2004b; Malhotra et al. 2012; Bajenoff et al. 2006; Roozendaal et al. 2009) that are distinguished from other lymph node cells by their expression of podoplanin (PDPN) and plate-derived growth factor receptor-α (PDGFRα), and lack of expression of CD31 and CD45 (Fletcher et al. 2015). They express many molecules that are common to fibroblasts, including desmin, vimentin, CD73, CD90, CD105, and α-smooth muscle actin (Link et al. 2007; Malhotra et al. 2012). In addition, FRCs express an immunologically relevant group of genes related to antigen presentation and cytokine response pathways (Malhotra et al. 2012). FRCs are functionally related to follicular dendritic cells (FDCs) (Heesters et al. 2014), which some consider to be one of the five subsets of FRCs (Jarjour et al. 2014).

Lymph nodes are immunological meeting places where B cells, T cells, DCs, macrophages and plasma cells congregate and move within a network composed of FRC and infiltrating lymphatics (Fletcher et al. 2015). The lymph node lattice guides soluble antigens in the incoming lymph from subcapsular sinuses to the T cell zones of the lymph nodes (Sixt et al. 2005) and facilitates interactions between T cells and antigen-presenting cells (Gretz et al. 1996; Balogh et al. 2002). Incoming naïve T cells immediately contact the FRC after entering lymph nodes via HEV, and actively migrate along the FRC network as they search for antigen (Bajenoff et al. 2006; Bajenoff et al. 2008). FRC produce IL-7, which is important in maintaining the $CD4^+$ memory T cell survival niche, as well as CCL19 and CCL21, which are important in positioning and motility of T cells (Luther et al. 2000; Okada and Cyster 2007; Link et al. 2007). The level of supportive factors produced by FRC function as a control on the level of circulating naïve T lymphocytes, which are produced in over-abundance and must be controlled. The intimate contact between FRCs and migrating lymphocytes puts FRCs in position to fundamentally regulate adaptive immunity (Malhotra et al. 2013).

Lymph nodes of mice in which FRC function has been ablated lose the ability to segregate T cells and B cells into their respective compartments, fail to maintain normal T cell numbers, and are incapable of mounting virus-specific CD4 and CD8 T cell responses (Cremasco et al. 2014; Denton et al. 2014). The failure to maintain T cell numbers is probably due to inadequate production of IL-7 when FRC populations are depleted (Link et al. 2007). Similar modulations of immune cell populations occurs when fibrosis damages the FRC network (Estes et al. 2008; Zeng et al. 2011). Fibrosis may be a permanent post-inflammatory transformation of a lymph node, thus the lymph node may be effectively incapacitated if severely damaged by inflammation. FRCs produce BAFF, the B cell survival factor, therefore disruption of FRCs also has a deleterious effect on germinal center formation and humoral immunity (Cremasco et al. 2014).

FRCs are key to re-establishment of lymph node homeostasis after damage to lymph node structure and function (Estes et al. 2015; Onder et al. 2012), which makes these cells attractive pharmacological targets. Fibrosis of lymph nodes, which occurs with HIV-1 and other infections, is a permanent alteration that can

have a major impact on future lymph node function. Anti-fibrotic measures may target various functions of FRCs, e.g., the pirfenidone (5-methyl-1-phenylpyridin-2-one) targeting of the TGFβ1 pathway to reduce extracellular matrix production by FRCs (Estes et al. 2015). FRCs administered as a cell therapy in animal models of sepsis and acute endotoxemia resulted in reduced mortality when administered soon after the septic insult (Fletcher et al. 2014).

Follicular dendritic cells (FDCs) in the stroma of secondary lymphoid organs provide the network that organizes the B cell follicles of germinal centers (Mueller and Germain 2009). In germinal centers the FCDs present antigen-containing complexes that are bound by low-affinity FC receptors for IgG and complement receptors to B cells. FDCs present the complexed antigen for a long period of time, and are important in the reconstitution of antigen responses, but the FDCs do not qualify as immunological memory cells as defined above. FDCs participate in the germinal center reaction, which results in the selection of B cells that produce high-affinity antibodies (Shiow et al. 2006).

Marginal reticular cells (MRCs) are a specialized subset of lymphoid stromal cells that have a role in the capture and delivery of antigens. They were first described in the marginal zone of the spleen, but functionally similar cells are present beneath the subcapsular sinuses of lymph nodes, particularly at the edges of lymphoid follicles, and in mucosa-associated lymphoid tissue such as Peyer's patches. The MRCs of lymph nodes provide structural support and produce chemokines that include CXCL13, a characteristic that is shared with the FDCs in the center of lymphoid follicles. They form a conduit that delivers antigens from the subcapsular sinuses of lymph nodes to the B cell follicles (Roozendaal et al. 2009). The MRCs of the spleen, along with marginal zone metallophilic macrophages, are also thought to have a role in delivery of antigens to B cell follicles in the spleen (Roozendaal et al. 2009; Junt et al. 2007). MRCs have many features in common with the lymph node stromal organizer (LTo) cells that are critical in the embryogenesis of lymph nodes (Yoshida et al. 2002; Katakai et al. 2008; Cupedo et al. 2004; White et al. 2007).

Red pulp fibroblasts are present in the non-lymphoid red pulp zone of the spleen. These cells are involved in the formation of splenic cords and providing direction to blood flow, assist in removal of effete red blood cells, and attract and retain macrophages and plasma cells. The splenic cords are composed of a compact network of fibroblasts and reticular fibers that have a critical role in filtering blood and providing a support scaffold for splenic macrophages. Red pulp fibroblasts express ICAM1 and CXCL12, which bind the LFA1 and CSCR4 expressed by plasma cells, thus serving to localize those antibody-producing cells in the spleen (Ellyard et al. 2005; van den Berg et al. 1993). In malaria infection or following exposure to endotoxins, the red pulp fibroblasts can fuse to form 'barrier cells' that restrict or alter blood flow.

Lymph node medullary fibroblasts, as the name implies, are limited to the medullary region of lymph nodes. They form a dense network of fibroblasts and reticular fibers in medullary cords, and a loose network within medullary sinuses (Willard-Mack 2006; Ushiki et al. 1995). They serve to attract and perhaps retain

macrophages, plasma cells, dendritic cells and mast cells. Expression of CXCL12 by lymph node medullary fibroblasts probably directs plasma cell localization to the medullary region of lymph nodes, similar to the effect of that chemkine in the spleen.

Vascular and lymphatic endothelial cells are abundant in SLOs, and have many functions other than fluid transport. Lymph nodes serve as hubs of antigen presentation where lymphocytes are primed or tolerized against antigens that are presented by APCs. The specialized HEVs of lymph nodes express peripheral node addressin (PNAD), which is a critical homing signal for entry of lymphocytes into lymph nodes. Lymphocytes continuously recirculate from the blood to lymph nodes and back to the blood as often as one or two times a day (Gowans 1959), thus allowing lymphocytes to search for the relatively rare cognate antigen that will result in their stimulation. Naïve T and B cells home to lymph nodes (Butcher and Picker 1996; von Andrian and Mempel 2003) and extravasate through the wall of HEVs via a multistep adhesion cascade (Gowans 1959; Marchesi and Gowans 1964; Girard and Springer 1995; Miyasaka and Tanaka 2004)). Mouse B cells spend approximately 24 h exploring the lymph node, while T cells spend only 8–12 in the lymph node (Tomura et al. 2008). If cognate antigen is not encountered, lymphocytes exit the lymph node via efferent lymphatics using the sphingosine-1-phosphate (S1P)-S1P receptor type 1(S1PR1) signalling pathway and return to the general circulation via the thoracic duct. HEVs are found in most secondary lymphoid organs, with the exception of the spleen (von Andrian and Mempel 2003; Girard and Springer 1995; Miyasaka and Tanaka 2004). In normal homeostasis HEVs are found only in lymphoid tissues, but they may develop in non-lymphoid tissues in association with chronic inflammatory diseases (Girard and Springer 1995; Drayton et al. 2006) and cancer (Martinet et al. 2011). In these latter situations the presence of HEVs is associated with a high level of lymphocyte infiltration into the tissues. The lymphocyte infiltration may be beneficial, as with some tumor-infiltrating lymphocytes (TILs), but may be detrimental to the host in situations such as autoimmune inflammatory diseases.

The initial interaction of lymphocytes with the endothelium of HEVs is mediated by the lymphocyte homing receptor L-selectin (CD62L) (Girard and Springer 1995; Rosen 2004). L-selectin recognizes a family of sulphated, fucosylated and sialylated mucin-like glycoproteins that are expressed by HEV endothelial cells. These interactions mediate sequential steps of rolling, sticking, crawling and transmigration that are similar to leukocyte emigration in inflammed tissues. However, following emigration the exiting lymphocytes do not travel through adjacent tissues to sites of inflammation. Instead, the lymphocytes accumulate in pockets at the base of the HEV endothelial cells. The accumulations of lymphocytes cause the endothelial cells of HEVs to bulge into the lumen, creating the ‘high endothelial’ phenotype (Girard et al. 2012).

Lymphatic endothelial cells (LECs) are the first cells that come into contact with peripheral antigens (Clement et al. 2011; Clement et al. 2010), immune cells (Randolph et al. 2005), cytokines and danger signals that are traveling from peripheral tissues to lymph nodes. The LECs have been shown to modulate dendritic cell

function (Jakubzick et al. 2006), present antigens to T cells on both MHC class I and class II molecules (Itano et al. 2003), and express immunomodulatory cytokines and receptors (Card et al. 2014). Lymphatic endothelial cells also express molecules that facilitate entry of lymphocytes into lymph nodes (Card et al. 2014).

In addition to providing navigational guidance and support for immune cells as they traverse lymph nodes, lymph node stromal cells directly participate in antigen presentation (Hirosue and Dubrot 2015). This function is essential for the induction of tolerance to peripherally-expressed antigens (Link et al. 2007; Bajenoff et al. 2006; Hammerschmidt et al. 2008; Molenaar et al. 2009). Peripheral tolerance induction in lymph nodes is mediated through stromal cell expression of autoimmune regulator (AIRE), which is also expressed by thymic epithelial cells that are involved in the generation of central tolerance (Gardner et al. 2008; van de Pavert and Mebius 2010).

Bone marrow stromal cells include mesenchymal stromal cells, endothelial cells, osteoblasts, and adipocytes which contribute significantly to marrow homeostasis. Mesenchymal stromal cells, also known as reticular cells, form a cellular network with an extracellular matrix composed primarily of collagen III (Bentley et al. 1981). This cell population is not sensitive to radiation, suggesting the cells are not actively replicating (Patt and Maloney 1975). The population of mesenchymal stromal cells produces a variety of mediators that are known to support the development of various hematopoietic cells.

Since the bone marrow does not have lymphatic vessels, the vascular endothelial cells lining the sinusoids, venules and arterioles constitute a regulatory point for entry of circulating cells entering the bone marrow (Pabst 2007). The endothelial cells lining the marrow sinusoids control the diameter of the vessel, which limits the distribution and velocity of blood flow in the marrow in a fashion similar to that seen in hepatic sinusoids (McCuskey 2000). The endothelial cells of the various vascular structures express different mediators that are involved in the development and egress of hematopoietic cell types. Sinusoidal endothelial cells are known to provide a survival niche for hematopoietic progenitor cells (Kopp et al. 2009; Kiel et al. 2005), and are thought to have a role in the regulation of peripheral B cell numbers by positioning immature B cells in the sinusoids and regulating their exit (Sapoznikov et al. 2008; Pereira et al. 2009).

Bone marrow stromal cells are involved in the generation and maintenance of memory $CD4^+$ T cells and memory plasma cells. The exact anatomic site of the conversion of antigen-dependent signaling to antigen-independent memory $CD4^+$ T cells is not known, but there is evidence this conversion takes place in the bone marrow, with support by mesenchymal stromal cells (Tokoyoda et al. 2010). The cellular proliferation of memory cytotoxic T cells takes place in the bone marrow, where the cell population is supported by IL-7-producing stromal cells (Parretta et al. 2005), but there is concern this proliferation is evidence of an extended antigen-dependent immune reaction that coincidentally involves the bone marrow rather than a true memory T cell population (Tokoyoda et al. 2010; Becker et al. 2005; Mazo et al. 2005). Memory B cells are thought to home to specific sites in the

spleen rather than the bone marrow (Mamani-Matsuda et al. 2008; Martinez-Gamboa et al. 2009; Dogan et al. 2009).

Plasma cells that are formed in secondary lymphoid organs (SLO) can be either short-lived or long-lived. The long-lived plasma cells translocate from the SLO to the bone marrow, where they associate with stromal cells that express CXC chemokine ligand 12 (CXCL12) and vascular cell-adhesion molecule 1 (VCAM1), differentiate into memory plasma cells, and persist in a nonproliferative resting state (Hargreaves et al. 2001; Hauser et al. 2002). The bone marrow stromal cells form dedicated niches that support the plasma cells and determine the quantity of subsequent memory responses. The long-lived plasma cells persist for an undefined period in the bone marrow, and constitute an independent compartment of humoral immunological memory (Radbruch et al. 2006; Manz et al. 1998; Slifka et al. 1998). Activated $CD4^+$ effector T_H cells also translocate to the bone marrow at the end of an immune response, dock with stromal cells expressing IL-7 and VCAM-1, and are maintained there as resting $CD4^+$ T_H cells (Tokoyoda et al. 2009b). It is probable that a similar situation exists for memory $CD8^+$ T cells and B cells (Tokoyoda et al. 2009a). These populations of memory cells should be considered when test articles in toxicology studies cause a pronounced reduction in bone marrow cellularity, which is often followed by microscopically apparent reversal after cessation of dosing. A return to microscopically normal cellularity does not necessarily mean the original bone marrow population has returned to full function, i.e., it may take some period of time to reconstitute the full repertoire of memory plasma cells in the marrow.

2.3 Cells of Tissues with Secondary Immune Functions

2.3.1 Liver

The liver is the primary hematopoietic organ during a major portion gestation, and retains many immune-related functions into adult life. Different immunological functions are attributable to the various cell populations of the liver, and are presented as such below. The liver is the organ that is most commonly injured by xenobiotics (Horner et al. 2013), thus there is a substantial potential for immunomodulation secondary to liver injury in toxicology studies.

The liver has extensive involvement in innate and adaptive immunity, which has been reviewed (Parker and Picut 2005, 2012) and is summarized as follows:

Innate Immunity Involvement

Production of acute phase proteins
Nonspecific phagocytosis
Nonspecific cell killing
Disposal of waste molecules

Adaptive Immunity Involvement

Deletion of activated T cells
Induction of tolerance to ingested and self antigens
Extrathymic proliferation of T cells
Disposal of waste molecules

2.3.1.1 Kupffer Cells

Nonspecific phagocytosis of particulate material is a major function of the innate immune system. Nonspecific phagocytosis in the liver is mediated primarily by Kupffer cells, which are present throughout the hepatic parenchyma. Kupffer cells in different areas of the hepatic lobules vary in population density, cytologic characteristics and physiologic functions. Various observations suggest a concentrated population of highly active Kupffer cells in the periportal region of the hepatic lobule, which is the first point of contact for in-coming, potentially pathogen-laden blood (Sleyster and Knook 1982).

Kupffer cells are a major cellular component of the liver, constituting 31% of the sinusoidal cell population, or $14–20 \times 10^6$ Kupffer cells per gram of tissue. Following pulse-labeling with latex particles, the population of latex-labeled Kupffer cells did not change over a period of 3 months, suggesting a long lifespan for these resident macrophages (Bouwens et al. 1986). Presence of the Fc receptor allows Kupffer cells to have a significant role in control of inflammatory and immunologic processes (Ravetch 1994). Kupffer cells have a low mitotic rate (0.06% after a 6-h arrest by vinblastine), thus have a low rate of labeling by ^{3H}thymidine (Bouwens et al. 1986).

The liver has been shown to be the primary site for removal of experimentally administered antigen and immune complexes. Soluble IgG complexes are eliminated from the circulation mainly by Kupffer cells and, to a lesser degree, sinusoidal endothelial cells, thus giving the liver a significant role in the control of inflammatory and immunologic processes. Uptake of immunoglobulin complexes is mediated by subtypes of the Fcγ receptor, principally Fcγ receptor IIB2 (FcγRIIB2) and Fcγ receptor III (FcγRIII) on Kupffer cells and sinusoidal endothelial cells. Kupffer cell recognition of the Fc domain of immunoglobulins results in nonspecific phagocytosis of immune complexes as well as antibody-coated particles such as microorganisms and eukaryotic cells. In addition to Fc receptors, Kupffer cells also have complement receptors for binding and phagocytosis of erythrocytes coated with complement fragments (Smedsrod et al. 1985b). The avid binding of immunoglobulin- or complement-coated erythrocytes allows Kupffer cells to have a major role in removal of erythrocytes from the circulation, resulting in the well-known Kupffer cell accumulation of iron-positive materials in disease processes that involve intravascular erythrolysis or erythrocyte sequestration.

2.3.1.2 Liver-adapted NK Cells ('pit cells') and NKT Cells

Pit cells are intrasinusoidal, liver-specific NK cells that are defined morphologically as large granular lymphocytes (LGLs) and functionally as liver-associated natural killer (NK) cells that are continually activated (Luo et al. 2001). The morphologic features of pit cells suggest they represent a more mature form of circulating NK cells (Nakatani et al. 2004). Pit cells are located inside the sinusoidal lumen, where they adhere to endothelial cells and Kupffer cells. Pit cells remain in the liver approximately 2 weeks and proliferate locally when stimulated by IL-2. They exhibit a high level of endogenous cytotoxicity against a variety of tumor cells, and act synergistically with Kupffer cells in tumor cell killing (Wisse et al. 1997).

NK cell killing of target cells is mediated via two major pathways: Fas/FasL and perforin/granzyme. The Fas/FasL pathway involves binding of the ligand, FasL, to the receptor Fas and subsequent activation of 'death domain' signaling elements, resulting in activation of the caspase cascade and apoptosis. The perforin/granzyme pathway involves perforin-mediated introduction of pores in the cell membrane and introduction of granzymes into the cytosol of the target cell. The perforin/granzyme pathway essentially constitutes a 'shortcut' that bypasses the death domain-containing signaling molecules on the surface of target cells and proceeds directly to the downstream caspase cascade. Granzyme B activates caspase-3, which then removes the propeptide of caspase-7, resulting in activation of the executioner caspase-7 (Yang et al. 1998).

Signaling molecules produced by Kupffer cells are known to influence Fas/FasL- or perforin/granzyme-mediated cell killing by NK and NKT cells. Interleukin-18, which was first identified as an interferon-gamma (IFNγ)-inducing factor produced by activated Kupffer cells, promotes Fas/FasL-mediated killing by NK cells (Tsutsui et al. 1996) and augments perforin/granzyme-mediated killing by NK-T cells (Dao et al. 1998). Kupffer cells also produce IL-12, which was first identified as 'NK cell stimulating factor'.

With increasing age there are changes in the hepatic population of NK cells. The primary tumor cell-killing NK cell subpopulation ($NK1^+$ TCR^{int}) in the liver increases until middle age and then declines, resulting in a reduction in this critical first-line defense against invading tumor cells at an age when tumor metastasis to the liver is most likely.

NKT cells are abundant in the liver (Nakatani et al. 2004), and it has been shown that NKT cells can develop extrathymically from liver precursors (Shimamura et al. 1997). This liver-resident, locally regenerating pool of rapid-response killing cells also has a significant role in defending the liver from invading tumor cells.

Innate immune functions of NK and NKT cells may be particularly important in invasion of the liver by metastatic colon carcinoma cells, and possibly infiltrative esophageal carcinoma cells (Wu et al. 2015). Highly malignant colon carcinoma cells often express FasL, which binds to Fas expressed by tumor-infiltrating lymphocytes (TILs), resulting in apoptosis of the anti-tumor TIL population (O'Connell et al. 2000; Ryan et al. 2006; Loose and Van de Wiele 2009), a process known as

'Fas counterattack'. In addition, FasL expression by infiltrating colon carcinoma cells binds to Fas expressed on the surface of hepatocytes. The Fas/FasL-mediated apoptosis of hepatocytes, which is also seen in some forms of viral hepatitis (Hayashi and Mita 1999), results in a nidus of necrotic hepatocytes that serves as fertile soil for tumor cell growth, with no resistance by TILs.

Not all features of the hepatic microenvironment are pro-apoptotic and anti-tumor. Hepatic sinusoidal endothelial cells express serine protease inhibitors 6 and 9 (SPI-6 and SPI-9), which inhibit the perforin/granzyme pathway and thus alter the liver microenvironment to hinder pit cell-mediated killing of metastatic tumor cells (Vermijlen et al. 2002).

2.3.1.3 Sinusoidal Endothelial Cells

Hepatic sinusoidal endothelial cells (SECs) have numerous functions in the innate and adaptive immune systems. SECs have a unique cell marker phenotype that would be consistent with myeloid lineage or dendritic cells, but available evidence indicates SECs are derived from hepatocyte precursors (O'Farrelly 2004). SECs may represent a population of organ-specific antigen-presenting cells. SECs have a voracious appetite for circulating molecules, to the degree that SECs are known as 'professional pinocytes'. Receptor-mediated endocytosis by SECs occurs primarily via four categories of surface receptors: collagen receptors, mannose receptors, scavenger/hyaluronan receptors, and Fc receptors.

Collagens are dynamic molecules that constitute a major component of the mammalian body. Formation and turnover of collagen results in the release of large quantities of collagen components into the circulation, and these must be cleared in order to prevent undesirable immunological consequences. SECs of rat liver express a type of receptor that specifically recognizes and mediates the endocytosis of collagen alpha 1 monomers and denatured collagen (gelatin) (Smedsrod et al. 1985a). However, clearance of all collagen products is not necessarily mediated via the collagen receptor on SECs. Circulating C-terminal propeptide of type I procollagen is cleared mainly via the SEC mannose receptor (Smedsrod et al. 1990) and clearance of NH_2-terminal propeptides of types I and III procollagen is a function of the SEC scavenger receptor (Melkko et al. 1994).

Carbohydrates function as labels that mark circulatory glycoproteins for rapid clearance. The mannose receptor on SEC, which recognizes terminal mannose residues on macromolecules, is an essential element in the regulation of serum glycoprotein homeostasis (Lee et al. 2002). Proteomic analysis of mice that were genetically deficient in mannose receptor revealed elevated levels of eight different lysosomal hydrolases as well as four additional proteins that are up-regulated during inflammation and wound healing, indicating that functional hepatic mannose receptor is important in the control and resolution of inflammation. SECs have a voracious appetite for monosylated molecules as well as extraordinarily rapid rate of internalization of those labeled molecules. The endocytotic rate constant for mannose receptor-mediated internalization of

ovalbumin by SECs is 4.12 min^{-1}, corresponding to a half-life of approximately 10 s. This is one of the fastest known rates of internalization of a receptor-ligand complex (Magnusson and Berg 1989).

SEC contain many lysosomal enzymes, with some enzyme levels higher than in professional phagocytes such as Kupffer cells (Knook and Sleyster 1980). Some of the SEC lysosomal enzyme content is a result of sequestration of enzymes from the circulation via attachment of the SEC mannose receptor to a terminal mannose on the enzymes (Smedsrod and Tollersrud 1995). In contrast to other engulfed macromolecules, lysosomal enzymes are preserved and remain physiologically active within SECs.

The scavenger receptor of SEC cells assists in the clearance of numerous physiological and foreign waste macromolecules from the blood, including molecular debris resulting from turnover of the extracellular matrix, intracellular macromolecules, modified serum proteins, and bacterial and fungal proteins. The scavenger system is highly conserved through evolution. Studies have shown that species from all seven vertebrate classes have a population of nonmacrophagic scavenger endothelial cells that eliminate soluble waste macromolecules from the blood, constituting an important part of the innate immune system (Seternes et al. 2002).

Hyaluronan is a widely distributed component of connective tissue, and blood levels of hyaluronan are increased in immune-mediated diseases as well as certain malignancies. The major route of clearance of hyaluronan is via the liver, where it is taken up predominantly by SECs. An increased level of circulating hyaluronan may result from increased connective tissue synthesis/destruction or impaired hepatic capacity for waste molecule removal, as occurs in hepatic cirrhosis. The hyaluronan receptor shares functional properties with the scavenger receptor family (McCourt et al. 1999).

The Fc receptor on SECs has specificity similar to the Fc receptor on Kupffer cells. Both cell types are capable of removal of waste immunoglobulins from the circulation, though Kupffer cells have the major activity. In addition to binding the Fc region of IgG, the Fc receptor on SECs is also capable of binding IgA and IgA complexes (Kuiper et al. 1994).

In addition to its role in disposal of signaling and effector molecules of inflammation, the liver serves to remove T cells that were activated by inflammation at sites distant from the liver. Leukocyte emigration in the liver occurs in sinusoids rather than post-capillary venules, and does not require the typical selectin-mediated rolling step that occurs in leukocyte emigration from post-capillary venules (Wong et al. 1997). Selectin-mediated rolling is not required because hemodynamic factors, coupled with Kupffer cell migration and leukocyte interactions with vessel walls, serve to slow the rate of blood flow through liver sinusoids. These alterations in rate of blood flow vary in different regions of the hepatic acinus, and vary between species (MacPhee et al. 1995). The end result is that leukocytes in hepatic sinusoids have extensive, 'slow-motion' exposure to high-affinity, integrin-type adhesion molecules without the need for the preliminary rolling step.

Endothelial cells in vessels at sites of peripheral inflammation typically have increased expression of adhesion molecules such as ICAM-1 and VCAM-1, which

serve to localize emigration of leukocytes to the site of inflammation. SECs have constitutively high expression of ICAM-1 and VCAM, which facilitates integrin-mediated T cell adhesion in the absence of an inflammatory reaction in the liver. Absence of co-stimulatory and helper molecules in the liver microenvironment results in apoptosis of the sequestered activated T cells ('death-by-neglect'. In addition to the death-by-neglect mechanism, galectin-1 produced by endothelial cells directly induces apoptosis in sequestered activated T cells (Perillo et al. 1995; Lotan et al. 1994; Rabinovich et al. 1998). As a result of these processes, the liver is regarded as a "sink" for activated T cells (Mehal et al. 1999).

Induction of tolerance to ingested and self-antigens.

Liver sinusoidal lining cells can take up, process and present antigen to T cells but, probably due to the lack of input from helper T cells, the end result is tolerance rather than immunity (Rubinstein et al. 1986, 1987; Limmer et al. 2000). This function of sinusoidal endothelial cells prevents immunologic reaction to the wide spectrum of potentially antigenic molecules that are assimilated from the gastrointestinal tract.

2.3.1.4 Hepatocytes

Hepatocytes have less direct involvement in immunological functions than Kupffer cells, pit cells, NK cells, NKT cells, and SEC, but hepatocytes nonetheless have some contributions to immunity. Chief among these are production of acute phase proteins and presentation of self-antigens.

Acute inflammation in mammals is associated with a transient increase in a group of circulating proteins that are collectively known as acute phase proteins. In non-clinical toxicology studies the acute phase reaction may occur with inflammatory reactions associated with catheterization for infusion studies, neoplasms, xenobiotic-associated tissue injury, or many nonspecific disease processes such as the inflammatory lesions on the feet of rats ('tarsal granulomas') in long-term studies or auricular chondritis associated with placement of ear tags.

The acute phase response is generated when focal injury at some extrahepatic location prompts local macrophages to release a first wave of cytokines that includes IL-1, tumor necrosis factor alpha (TNFα), and a small amount of IL-6. Absorption of the first wave of cytokines into surrounding cells is followed by a second wave cytokine release, including a large amount of IL-6 that promotes massive production of acute phase proteins by hepatocytes (Fey et al. 1994). This includes serum amyloid A protein (SAA), fibrinogen, C-reactive protein (CRP), haptoglobin, complement factors C3 and C9, hemopexin, ceruloplasmin, $_{\alpha 2}$-macroglobulin, CD14, $_{\alpha 1}$-antichymotrypsin (ACT), $_{\alpha 1}$-cysteine proteinase inhibitor ($_{\alpha 1}$CPI), and $_{\alpha 1}$-antitrypsin (AAT) (Fey et al. 1994). Circulating acute phase proteins are responsible for many of the systemic effects of inflammation, which are largely aimed at preparing the body for resistance to systemic invasion and facilitating local resistance to

pathogens. The acute phase proteinase inhibitors (e.g., AAT, ACT, $_{\alpha 1}$CPI, and $_{\alpha 2}$M) reduce tissue damage due to proteinases that are released by dead or dying cells. Hemopexin and haptoglobin bind to heme and globin, respectively, which may be released by erythrolysis in inflammatory lesions. SAA and CRP have functions which suggest a scavenger role, but no single function has been identified that would explain the marked (up to 1000×) increase of these proteins in an acute phase reaction.

CRP assays are sometimes included in non-clinical toxicology protocols, therefore, that acute phase reactant warrants specific mention. CRP acts as an opsonizing agent, activates complement, binds to IgG receptors on mammalian cells and phosphocholine in bacterial membranes, and recognizes nuclear constituents in damaged cells. CRP has been shown to be a reliable indicator of inflammation associated with atherogenesis and other inflammatory disease processes in humans (Dupuy et al. 2003), but is not a reliable indicator of inflammation in all species. Serum haptoglobin level is a better indicator of systemic inflammation in swine, where it has been used to monitor overall swine herd health (Chen et al. 2003), and serum levels of $_{\alpha 2}$–macroglobulin, haptoglobin or fibrinogen are more accurate than CRP levels as indicators of inflammation in laboratory rats (Dasu et al. 2004; Chen et al. 2003; Giffen et al. 2003).

In addition to their extensive involvement in metabolism, detoxification and elimination, hepatocytes also serve as antigen-presenting cells. Extension of hepatocellular microvilli through intercellular junctions between SEC allows direct contact between hepatocytes and naïve $CD8^+$ T cells in sinusoids. However, T cell activation by hepatocytes leads to premature T cell death or tolerance rather than the formation of fully competent CT. Apoptosis of hepatocyte-activated T cells is an example of 'death by neglect' resulting from absence of an effective co-stimulatory signal. Apoptosis of hepatocyte-activated T cells is also induced by galectin-1 produced by SEC (Perillo et al. 1995).

Production of acute phase proteins has historically been considered as a function of hepatocytes, but a similar spectrum of proteins is produced during involution of the mammary gland following lactation and the uterus following parturition (Stein et al. 2004). The possible involvement of an acute phase reaction must be considered when unexplained serum chemistry and/or coagulation values are encountered in non-clinical toxicology studies, particularly reproductive toxicology studies.

2.3.1.5 Intrahepatic Dendritic Cells

Hepatic sinusoids serve to select and concentrate DCs as they arrive from the intestine via the portal circulation, and the DCs mature as they migrate from the portal vein entry point to the central vein exit. Mature DC near the center of the classical hepatic lobule traverse the space to Disse to enter the hepatic lymph system (Sato et al. 1998), eventually reaching the hepatic lymph nodes where antigens of intestinal or hepatic origin are presented to T cells.

2.3.2 Intestinal Cells

2.3.2.1 Mucosal Epithelial Cells

The mucosal immune system of the intestine (see Volume 2, Chap. 4, Mucosa-associated Lymphoid Tissue) includes inducible, reversible elements derived from cryptopatches, the latter being clusters of dendritic cells and lymphoid cells similar to the LTi cells that are involved in the generation of lymph nodes and Peyer's patches (Eberl 2005; Mebius 2003; Kanamori et al. 1996). These elements are part of a delicate balance between the intestinal microbiome and intestinal immunity, in which intestinal organisms do not invade the epithelial structures of the intestine and the intestinal immune system does not destroy the commensal organisms of the intestinal microbiome (Guarner and Malagelada 2003; Hooper and Gordon 2001; McCracken and Lorenz 2001). In addition to their participation in formation of lymphoid structures and adaptive immunity, LTi-like cells of the intestine have a direct effect on innate immunity in the intestine. LTi-like cells of the intestine produce IL-22, which is necessary for production of antimicrobial peptides and maintenance of epithelial barrier function in the intestine (Ouyang et al. 2008; Zheng et al. 2008). Presence of commensal organisms in the intestine is necessary for production of IL-22 by LTi-like cells (Sanos et al. 2009; Satoh-Takayama et al. 2008). This latter activity is of particular interest in the interpretation of toxicology studies, as it represents a direct pathway by which test article-related alterations in the commensal organism population could alter the structural integrity and barrier function of the intestine.

2.3.2.2 Tuft Cells

Tuft cells comprise a minor population of intestinal epithelial cells that are thought to represent quiescent stem cells, but there is evidence that tuft cells also serve a sentinels in the gut epithelium that promote type 2 immunity to intestinal protozoan and metazoan parasites (Gerbe et al. 2011; Westphalen et al. 2014; McKenzie et al. 1998; Howitt et al. 2016). Tuft cells have taste-chemosensory apparatus, but it is not known whether tuft cells sense microbiota by means of taste sensation. The tuft cell population has been shown to expand in response to presence of some microbiota, e.g., trichomonads (Urban et al. 1998) and various helminths (Howitt et al. 2016). Tuft cells are the main intestinal source of parasite-induced IL-25, which indirectly induces tuft cell expansion by promoting IL-13 production by innate lymphoid cells in the gut, thus forming a positive feedback cycle that increases the population of tuft cells in the presence of intestinal helminths.

2.3.2.3 Paneth Cells

The innate mucosal barrier of the intestine consists of mucosal epithelial cells, mucins, IgA and antimicrobial effector molecules (Salzman 2010). In intact hosts, the intestinal microbiome is critical to host protection by forming a mucosal barrier

that prevents invasion by potential pathogens (Hapfelmeier et al. 2010). This is accomplished primarily through the process of "colonization resistance", whereby indigenous microbiota compete with potential pathogens for scarce supplies of essential microbial nutrients. Breakdown in this system is a facet in the pathogenesis of human disease such as obesity (Ley et al. 2006), diabetes (Wen et al. 2008), irritable bowel syndrome (Parkes et al. 2008), and inflammatory bowel disease (Sartor 2008).

While it is clear that the intestinal microbiome has a direct influence on immunological and other functions of the host, less is known about influences of the host on the intestinal microbiome. Early studies indicated that intestinal IgA influenced intestinal microbiome components (Suzuki et al. 2004). More recent evidence indicates that defensins produced by host cells in the intestine may have a role in maintaining the population of microbes in the intestinal microbiome (Wilson et al. 1999; Salzman et al. 2003). Defensins are a class of cationic antimicrobial peptides that have broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, fungi and viruses (Zasloff 2002; Ouellette et al. 1994). Of the three classes (α, β, and θ) of defensins that have been identified in mammals, only the α and β classes have been identified in mice and humans (Salzman 2010). In humans, α defensins are found in neutrophils and Paneth cells, while in mice the production of α defensins is restricted entirely to Paneth cells.

Paneth cells are derived from crypt stem cells (Porter et al. 2002), and in adults are located at the deepest aspects of the crypts of Lieberkuhn (Fig. 2.2). Paneth cells produce a number of immunologically active molecules in addition to defensins, including secretory phospholipase A2, TNFα, RegIIγ, lysozyme, and angiogenins

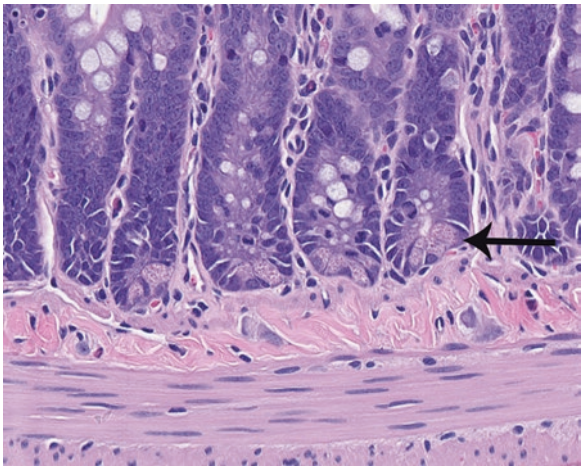

Fig. 2.2 The deep aspects of the crypts of Lieberkuhn of the jejunum of a young adult rat contain multiple Paneth cells (*arrow*) that have prominent eosinophilic cytoplasmic granules. Paneth cells produce a number of immunologically active molecules, including defensins that modulate components of the intestinal microbiome. Changes in these microbiome components have downstream effects on systemic adaptive immunity. H&E stain, 40× objective magnification

(Salzman 2010; Cash et al. 2006; Hooper et al. 2003). Some of these molecules are produced independently of intestinal microbes, while production of other molecules depends on the intestinal microbiome as a stimulus for production. Defensins produced by Paneth cells have a direct impact on the relative abundance of the various bacterial groups of the intestinal lumen, with a specific effect on the population of segmented filamentous bacteria (SFB) (Salzman et al. 2010). SFB, which are members of the Firmicute phylum, are one of the few members of the commensal flora that directly contact intestinal epithelial cells (Snel et al. 1995). SFB are highly sensitive to exogenous antibiotics (Croswell et al. 2009), and are also regulated by IgA (Suzuki et al. 2004). SFB are potentent stimulators of adaptive immune responses, and are known to stimulate the expression of IL-17A by $CD4^+$ T cells (T_H17 cells) (Ivanov et al. 2009; Gaboriau-Routhiau et al. 2009). These connections between Paneth cells, defensins, SFB, and adaptive immune responses must be considered in non-clinical toxicology studies that involve xenobiotic-related alterations in components of the intestinal microbiome or Paneth cell populations. See Volume 1, Chap. 7, Sect. 7.5.9 for information regarding interactions between antimicrobial xenobiotics, adaptive immune responses, and development of *Candida albicans* infections.

2.3.2.4 NK Cells/Large Granule Leukocytes

Histologic examination of the intestine of rats and other species commonly reveals the presence of individualized cells, other than eosinophils, that contain eosinophilic cytoplasmic granules or globules. One population of cells, typically located within the superficial epithelium, has small cytoplasmic granules. A second population of cells, located in both the lamina propria and the superficial epithelium, typically have a single large eosinophilic structure (globule). The exact genesis and nature of these cells is unclear, but available information suggests these cells belong to at least two biologically distinctive populations of mucosal cells with eosinophilic cytoplasmic granules/globules.

Mucosal mast cells of rats contain a granule-specific proteinase, RMCPII (Woodbury et al. 1978), and this enzyme has also been detected in a small proportion of globule leukocytes (GL) of parasitized rats (Woodbury and Miller 1982). RMCPII was detected by immunohistochemistry within mucosal mast cells and globule leukocytes, but not the granular intraepithelial lymphocytes that are present in the mucosal epithelium of the intestine of rats (Huntley et al. 1984). These observations suggest that mucosal mast cells and GL are of a common lineage, with granular intraepithelial lymphocytes being of a separate cellular lineage (Huntley et al. 1984). The exact nature of the granular intraepithelial lymphocytes is unclear, but the cells have some similarities to NK cells or cytotoxic T lymphocytes.

A study of the postnatal development of intestinal GL populations in rats revealed a substantial population of GLs in newborn rats, with no evidence of an associated infection (Ikeda and Yamashina 1993). The intestinal GL population decreased

markedly to reach the adult level by the fourth postnatal week. Ultrastructurally, the GLs had unique paracrystalline structures in the cytoplasmic globules. Administration of dexamethasone to the developing rats resulted in degranulaton of GLs. Observations in this study suggested that (1) GLs and mucosal mast cells (MMC) are derived from a common progenitor cell, (2) immature GLs migrate from the intestinal lamina propria into the epithelium to mature and proliferate, and (3) the immature GLs have specific functions during the neonatal period.

A study of VEGF production in the respiratory and gastrointestinal tracts of rats revealed solitary cells with VEGF immunoreactivity scattered in the epithelium of both the respiratory and digestive tracts (Fan and Iseki 1999). VEGF-positive cells in the respiratory tract were identified as GLs based on their distinctive ultrastructural features, and VEGF immunoreactivity in the respiratory tract was localized exclusively in GLs. VEGF-positive cells in the small intestine had morphological features similar to those of the respiratory tract, and were considered to be a form of mucosal mast cells (MMC). GL/MMC in the respiratory and intestinal mucosa had little immunoreactivity to histamine, as compared to the strong histamine immunoreactivity of the connective tissue mast cells located in the submucosa of the intestine. The GL/MMC population had strong immunoreactivity for VEGF, while the connective tissue mast cell population had no VEGF immunoreactivity. These observations suggest that GL and MMC in the respiratory and intestinal mucosa of rats have functional similarities and shared origin, and that VEGF immunoreactivity can be used as a marker for MMC. It was proposed that GL/MMC are involved in the paracrine regulation of permeability of nearby microvessels in the respiratory and intestinal tracts.

In a study of six intestinal leukocyte populations in mice following experimentally induced infection with *Strongyloides ratti*, the GL population paralleled the increase in mast cell populations seen in the initial stages of the infection (Carroll et al. 1984). Increased GL populations are also known to occur with intestinal parasitism in various domestic animals (Silva et al. 2012). One could speculate that increased GL/MMC populations seen in neonatal and parasitized animals could represent similar immunological reactions to newly encountered antigens from ingested nutrients or parasites.

Oral administration of polyetheylene glycol 400 (PEG 400), which is used as a vehicle in non-clinical toxicology studies, resulted in infiltrations of eosinophils and GLs in the glandular gastric mucosa of rats (Ueda et al. 2011). The cellular infiltrations were most prominent near the delimiting ridge that demarcates the boundary between the glandular and non-glandular regions of the stomach of rats. Given the common use of this vehicle material, toxicologic pathologists should be aware of this association between GL populations and PEG 400 administration.

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Chapter 3
Signaling and Effector Molecules in Immunity

Michael C. Milone

Abstract Initiation of an innate and/or adaptive immune response involves the coordinated function of multiple cells as well as numerous signaling and effector molecules, all supported by a stromal framework that may provide additional immunomodulatory signals. Signaling and effector molecules of the immune system have been the focus of intense investigation in recent decades, resulting in a massive accumulation of relevant information. The aim of this chapter is to provide an overview of the major groups of signaling and effector molecules, and to provide a general framework whereby these molecules function in the initiation, modulation, and dissolution of immune responses. Particular interest is given to those molecules and processes that represent targets for possible pharmacological intervention.

Keywords Cytokine • Chemokine • Interferon • Tumor necrosis factor (TNF) • IL-1 • T cell receptor (TCR) • B cell receptor (BCR) • Antibody • Fc receptor • Complement

3.1 Introduction

The immune system has evolved in lock step with the evolution of animals. Even the oldest known metazoans, the sponges, possess primitive immunity that provides protection against invading pathogens and displays histocompatibility that is in many ways similar to that observed in vertebrates (Paul 2013). The vertebrate immune system is a highly complex system that is comprised of physical barriers, cells and effector molecules that operate in all body compartments. It has evolved to be a highly effective defense system that is critical for maintaining tissue homeostasis. However, regulating the function of this complex system is essential for an organism's survival. Failure of immunity results in either uncontrolled infection or self-destruction by autoimmunity.

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Enormous advances in our understanding of immunity and ways to manipulate it have occurred over the past quarter century with the advent of molecular biology and the genetic manipulation of cells and organisms. These studies have uncovered a wide array of molecules used by the immune system to recognize and eliminate pathogens. These recognition and effector molecules are combined with networks of cytokines, chemokines and small molecule messengers to facilitate communication between immune cells that are separated in both space and time and orchestrate an effective immune response. This chapter provides a general overview of the major classes of molecules involved in immune recognition and effector function with a particular focus on those of current pharmaceutical interest. While the chapter is organized into innate and adaptive mechanisms, it is important to recognize that these immune pathways intersect at many places and exert great influence on each other. Successful immunity is in fact dependent upon this crosstalk.

3.1.1 Innate Immune Recognition

Pathogen recognition is critical to immunity and host survival. As a result, mammals have evolved a wide range of mechanisms to recognize pathogenic microorganisms both directly and through the tissue damage associated with infection. While recognition of pathogenic-specific antigens through the B-cell receptor (BCR) and T-cell receptor (TCR) are critical to adaptive immunity and long-lasting immunologic memory as discussed later in this chapter, the adaptive immune response depends in great part upon the inflammatory signals that occur early during infection to differentiate pathogenic antigens from endogenous self-antigens. Inflammation induced by damage-associated signals has also been implicated in a number of non-pathogen associated inflammatory disorders including gout and asbestosis. This apparent dependency upon damage and inflammation to generate robust adaptive immunity has been called the “danger hypothesis” due to the observation that antigens presented in the absence of significant inflammation often leads to tolerance rather than immunity. While this may be an over-simplified model of immunity, there are undoubtedly important relationships between innate immune recognition and inflammation, the generation of robust adaptive immunity and unwanted immunopathology. Understanding these pathways of innate pathogen recognition and their dysregulation in inflammatory diseases represents an important area of research and development unveiling critical control points for targeted therapeutics. Effective activation of these pathways also likely represents the key to effective vaccines.

3.1.1.1 Pattern Recognition Receptors (PRRs)

Charles Janeway, Jr. proposed the concept of microbial PRRs over 25 years ago in an attempt to reconcile the fact that adaptive, clonally-selected immunity to model antigens such as haptenized proteins often requires the use of potent adjuvants derived from microbes, what he termed “the immunologist’s dirty little secret”.

Table 3.1 Some cytokines and associated JAK-STAT signaling

Cytokine	JAKs	STATs
IFN-γ	JAK1, JAK2	STAT1
IFN-α/β	JAK1, TYK2	STAT1, STAT2, STAT3
IL-6	JAK1, JAK2, TYK2	STAT3
IL-12	JAK2, TYK2	STAT4
IL2	JAK1, JAK3	STAT5A/STAT5B
IL4	JAK1, JAK3*, TYK2*	STAT6

*JAK3 activation depends upon γ_c receptor expression. IL-4Rα/IL-13Rα1 receptor heterodimers (so called IL-4R type II) activate TYK2 instead of JAK3 (Wills-Karp and Finkelman 2008)

His idea turned out to be incredibly prescient, and at least five distinct families of PRRs have been described over the intervening quarter century since Janeway proposed his hypothesis (Janeway 1989). These receptors, shown in Table 3.1 recognize a range of microbial-derived ligands including structural components of bacteria, fungi and viruses, so called pathogen-associated molecular patterns (PAMPs) along with endogenous proteins that are release or exposed following cellular and tissue in injury, so called damage-associated molecular patterns (DAMPs). Importantly, these PRRs provide the central link between pathogen-associated molecules and signals that trigger the production of inflammatory cytokines (e.g. IL-1, IL-18 and the interferons) and enhance antigen presentation that are critical to the initiation of protective innate and adaptive immunity. These receptors play critical roles in vaccination serving as the primary mechanism for recognition and response to adjuvant. It is likely that the dysregulation of PRRs and/or their intracellular signaling also contribute to the pathogenesis of inflammatory disease and autoimmunity.

3.1.1.2 Toll-Like Receptors (TLRs)

One of the earliest defined PRRs, the TLRs are a family of single pass membrane receptors that are comprised of an extracellular domain with leucine rich repeat (LLR) domains involved in ligand binding and specificity, a transmembrane domain and a cytoplasmic domain containing the conserved Toll-like/IL-1 Receptor/Resistance (TIR) that binds adapter molecules that link receptor ligand binding to downstream cellular signaling. The TLRs derive their name from their similarity to the Toll receptor in *Drosophila melanogaster*, which plays a role in innate immunity to fungi in the fly by stimulating the production of anti-microbial peptides (AMPs) that are discussed in more detail latter in this chapter.

TLR4, expressed predominantly by cells of the myeloid lineage including monocytes, macrophages, dendritic cells and granulocytes, was the first TLR identified to play a role in immunity in mammals, and it is the most well understood member of this family of PRRs. TLR4 is part of a multicomponent receptor complex that recognizes the PAMP within lipopolysaccharide (LPS) endotoxin from bacteria. This recognition is essential to the inflammatory response associated with bacterial infection, and inactivating mutations of TLR4 result in mice that are resistant to LPS-induced septic shock

(Poltorak et al. 1998). TLR4 mediates its recognition of LPS in conjunction with soluble LPS-binding protein (LBP), CD14 and myeloid differentiation factor 2 (MD-2). Using mechanisms that are still largely unknown, LBP binding to LPS on the bacterial outer membrane transfers LPS to the CD14, MD-2 protein complex on monocytes. The binding of the lipid A moiety from LPS to the hydrophobic pockets of MD-2 leads to the dimerization of TLR4. This receptor dimerization triggers activation of a number of downstream signaling pathways including NF-κB and MAP kinase pathways that are initiated by the MyD88 adaptor molecule (Klett et al. 2014). While the LPS-mediated activation of TLR4 is the most well characterized, TLR4 appears to play a role in recognition of numerous other pathogen- or stress-associated ligands including viral envelope proteins (Kurt-Jones et al. 2000), heat shock proteins (e.g. HSP70) (Vabulas et al. 2002), HMGB1 and beta-defensins (Biragyn et al. 2002). Upon its ligation, TLR4, is a potent inducer of the inflammatory cytokines, IL-1, IL-6 and IL-8 as well as the B7.1 (CD80) molecule that is a critical ligand for CD28, which is required during antigen presentation for the initiation of T cell-mediated immunity (Medzhitov et al. 1997).

Ten different TLRs have been described in humans and 12 in mice. All deliver signals to cells through similar MyD88 and the related molecule TIRAP. TLR1, TLR2, TLR5 and TLR6 are expressed at the plasma membrane. These receptors bind a range of molecules including cell wall components from gram positive and gram negative bacteria (TLR1, TLR2 and TLR6) and bacterial flagellin (TLR5) similar to TLR4. TLR3, TLR7, TLR8 and TLR9 are localized to the endosomal compartment of the cell, and primarily recognize nucleic acid. Nucleic acids are ubiquitous within the extracellular environment. TLR9 was originally identified as a receptor that preferentially recognizes non-methylated cytosine and guanine (CpG) motifs, which are commonly found in bacteria in contrast to CpG sequences in mammals that are generally methylated. This recognition provides a limited degree of specificity for non-self, but is hardly absolute. The internal localization or compartmentalization of the nucleic acid-sensing TLRs is likely an additional, important mechanism by which nucleic acid-sensing TLRs maintain their specificity towards pathogens and damage. TLR9 in fact restricts is recognition activity to lysosomal-endosomal compartment through an N-terminal domain that restricts ligand binding and is cleaved by lysosomal protease. A number of mechanisms, some poorly understood, provide for clearance of extracellular DNA from dying cells and selective trafficking of exogenous nucleic acid to the compartments within the cell where TLRs might inadvertently recognize this self-derived nucleic acid. The lethal inflammatory disease that occurs in mice expressing a mutant TLR9 that does not require cleavage for functional activation illustrates the critical importance of receptor compartmentalization (Mouchess et al. 2011).

Despite a great deal of overlap in the TLRs between mice and humans, it is also important to recognize that there are in fact differences in TLR expression across species. For example, mice express TLR11, which recognizes a profiling-like molecule from the pathogen, *Toxoplasma gondii* as well as components of uropathogenic *Escherichia coli* bacteria. While the TLR11 gene is present in primates, it contains several premature stop codons in humans that prevent its expression (Roach et al. 2005). Given the importance of these innate PRRs in the recognition of pathogens and tissue damage, it is possible that these species differences might contribute

to altered inflammatory responses. These are therefore important considerations when using rodents for research and toxicology studies.

3.1.1.3 PRRs Beyond the TLRs

While TLRs represent one of the most well studied of the PRRs, at least 4 additional, general groups of PRRs have been described. These include the dectin (also known as C-type lectin domain [CTLD]) receptors, the nucleotide-binding domain, leucine-rich repeat-containing receptors (NLRs, or so called NOD-like receptors), RIG-1-like receptors (RLRs) and the AIM2-like receptors (ALRs). Each of these receptor families plays an important role in innate immunity. A review of these receptors is well beyond the scope of this chapter. Readers are instead referred to recent review articles describing the PRR families (Brubaker et al. 2015).

The dectins are comprised of a diverse group of receptors that recognize a wide range of microorganisms through their conserved CTLD that exhibits calcium-dependent binding to polysaccharide moieties. Some are soluble and some are membrane bound. While most dectins provide opsonin activity without activating signaling pathways, some such as dectin-1 (CLEC7A) that recognizes fungal β-1,3-glucans or dectin-2 (CLEC6A) that recognizes fungal α-mannans are capable of trigger an inflammatory signal through a Syk-dependent signaling pathway that uses the immunotyrosine-based activation motif (ITAM) found in many immunoreceptors including T-cell receptor (TCR) and B-cell receptor (BCR) complexes that are critical for adaptive immunity.

The prototypic NLRs, NOD1 and NOD2, in contrast to the TLRs and dectins, are found within the cytosolic compartment of cells. These receptors bind short peptide-like components of bacterial cell walls such as γD-glutamyl-meso-diaminopimelic acid or muramyl dipeptide, and activate a conserved signaling pathway through their caspase recruitment domain (CARD) and RIPK2 that results in NF-κB and MAPK activation similar to the TLRs. In addition to playing an important role in inflammatory signaling, these receptors also restrict intracellular bacterial pathogens by promoting their incorporation into autophagosomes that promote their elimination.

The cytoplasmic contains a number of other PRRs beyond the NLRs that survey this cellular compartment for nucleic acid as many pathogens such as viruses gain entry to the cytoplasm. One of the best characterized of these sensors in RIG-1 and RLRs. These receptors have evolved several mechanisms to distinguish endogenous cellular RNA from viral RNA including the recognition of long double stranded RNA (dsRNA) found in the genomes of reovirus or specific viral sequences such as the poly-uridine region of hepatitis C virus. RLRs have complex signaling that requires spatial localization to the surfaces of mitochondria and peroxisomes in order to stimulate the mitochondrial anti-viral signaling (MAVS) protein and tumor necrosis factor (TNF) receptor-associated factors (TRAFs) required for activation of NF-kB, the kinase TBK1 and its target, interferon regulatory factor 3 (IRF-3) required to drive type I and type III interferon (IFN) production. IFNs are important cytokines that mediate potent anti-viral effects as discussed in more detail below.

More recently, a cytoplasmic sensor termed STING (stimulator of IFN genes) was identified as a molecule plays important roles in the recognition of both viruses through its cooperation with IFI16 and DDX41 molecules that bind DNA and bacteria through STING's direct binding of cyclic dinucleotides (CDNs) that serve as second messenger molecules in bacteria. STING activates type I and type III IFN expression through activation of IRF3. The mechanism of IRF3 activation, similar to RIG-1, appears to also involve MAVS. The flavonoid compound 5,6-dimethylxanthenone-4-acetic acid (DXMAA), which was previously demonstrated to have potent anti-tumor activity in mouse models of cancer through enhancement of CD8+ T cell immunity, was recently identified to be a potent STING agonist. Unfortunately, human clinical trials of DXMAA failed to demonstrate an anticancer benefit; however, recent studies have demonstrated that DXMAA binds to mouse STING, but not to the human STING receptor. Due to the marked effects of STING agonists in murine models, efforts to generate potent a STING agonist for humans is an active area of investigation. Nevertheless, these results again illustrate that there are important differences between immunity between rodents and humans, which represents an important challenge to the use of rodents as models of human inflammatory disease and immunity.

3.1.1.4 The "Inflammasome" as a Unique Signaling Complex Driving Inflammation

Fever is one of the hallmarks of inflammation. In the search for mediators of the febrile response, interleukin-1 (IL-1) was identified as one of the endogenous pyrogens released by cells capable of inducing fever through effects on the hypothalamus along with potent effects on endothelium that lead to leukocyte adhesion, vascular permeability and vasodilation. Subsequently, a family of eleven IL-1-like molecules has been described that include the cytokines IL-18, IL-33 and IL-36. One of the conserved structural features of this cytokine family is the lack of a signal peptide for secretion. In some cases, these inflammatory cytokines such as IL-33 are localized to the cytoplasm or nucleus of cells, and IL-33 is released following necrotic cell death forming a type of non-silent cell death that aids in the triggering of immunity. The mechanism by which members of this family are secreted from non-dying cells remains unclear. These molecules are also produced as precursor proteins with an N-terminal amino acid sequence that is processed into a mature protein by protease cleavage. In the case of IL-33, this cleavage leads to the inactivation of the protein's biologic activity. In contrast, IL-1β and IL-18, the two most potent inflammatory members of this family are transcriptionally activated by PAMPs and DAMPs that drive NF-κB signaling (see below), but these proteins require the additional cleavage of their precursor proteins to generate mature proteins with biological activity. The regulation of IL-1β and IL-18 at both transcriptional and post-transcriptional levels therefore represents a critical safety feature in the control of these important inflammatory mediators.

Efforts to identify the enzymes responsible for IL-1β cleavage and activation uncovered an enzyme that was itself a zymogen requiring cleavage for enzymatic

activity. This IL-1β-converting enzyme (ICE, now generally referred to as caspase-1) was found to be similar to the ced-3 gene product in *Caenorhabditis elegans,* a member of a family of cysteine proteases called caspases that play important roles in programmed cell death. Similar to other members of the caspase family, caspase-1 contains a conserved protein interaction domain termed the caspase activation and recruitment domain (CARD). Caspase-1 through CARD-CARD interactions becomes incorporated into a large macromolecular complex that is capable of activating and recruiting the proteases necessary for cleavage of precursor caspase-1 into its matur `e form that activates IL-1β. This macromolecular complex has been termed an inflammasome. Inflammasomes generally contain a sensor protein, either members of the NLR or ALR families that detects PAMPs and DAMPs, an adaptor protein that provides a scaffold for assembly and recruitment of caspase-1 into the complex.

In canonical inflammasome assembly, DAMPs or PAMPs drive the activation of NLRs or ALRs. These PRRs form oligomers through interactions between conserved domains such as the NACHT domain in NLRs or the HIN-200 domains within the ALRs. Following oligomerization, the adaptor molecule ASC (apoptosis-associated, speck-like protein containing a CARD) is recruited into the growing complex through its pyrin domain (PYD) that interacts with PYD domains present with the NLRs and ALRs. Caspase-1 is subsequently recruited into the inflammasome through CARD-CARD interactions with ASC, and caspase-1 oligomerization leads to auto-cleavage and transformation into a potent IL-1β converting enzyme complex. NLRP3 is one of the best-characterized NLRs involved in inflammasome signaling. NLRP3 is activated by a diverse array of signals that include viral, bacterial and fungal pathogens as well as crystalline substances such as uric acid, alum and silica crystals; however, the mechanism by which these diverse stimuli lead to NLR activation is still largely unknown. Ligand binding directly by the NLRs may be involved in activation. These inflammasomes can also be activated by cellular perturbations such as potassium efflux from the cell, reactive oxygen species and cathepsin release from the phago-lysosomal compartment. These signals suggest a model of cellular injury as a common mechanism for inflammasome activation. The importance of inflammasome-mediated activation of caspase-1 is illustrated by the increased susceptibility of mice lacking caspase-1 to a range of pathogens including *Francisella tularensis* (Mariathasan et al. 2005; Fernandes-Alnemri et al. 2010), *Salmonella* sp.(Raupach et al. 2006; Lara-Tejero et al. 2006), *Pseudomonas aeruginosa* (Sutterwala et al. 2007) and influenza virus (Ichinohe et al. 2009).

While there is abundant support for a central role of inflammasomes in IL-1β and IL-18 activation, the inflammasome model fails to account for the severe IL-1β-dependent inflammation that occurs in caspase-1-deficient mice following chemically-induced or crystal-induced tissue injury (Fantuzzi et al. 1997a, b; Guma et al. 2009). Disseminated infection by *Candida albicans* is also IL-1β-dependent, but caspase-1-independent indicating that other mechanisms for IL-1β and IL-18 activation must exist (Mencacci et al. 2000). There is increasing evidence that additional enzymes can cleave IL-1β and IL18 precursors into their biologically active form. These include serine proteases such as proteinase 3 (PR3) and elastase from neutrophils (Coeshott et al. 1999; Sugawara et al. 2001; Joosten et al. 2009) and chymase from mast cells (Omoto et al. 2006). The metalloproteinases, Meprin α and

Meprin β have also been shown to process IL-1β into a biologically active form (Herzog et al. 2009). Understanding the non-canonical signaling pathways that lead to activation of these potent inflammatory cytokines will therefore be necessary in order to develop effective therapies that control inflammatory diseases.

3.1.1.5 NF-κB: A Master Regulator of Inflammation

Since the first report by Sen and Baltimore in 1986 of nuclear factors in B cells that associate with a sequence in the enhancer region of the kappa light chain gene, more than 25,000 papers have been published on these nuclear factors, which have been termed NF-κB. NF-kB family members are expressed across a range of animal species including Cnidarians, insects and mammals. They play critical roles in the cellular response to stress as well as inflammation, regulating genes involved in cellular metabolism, differentiation and proliferation. Defects in NF-kB signaling are also linked to a variety of diseases including cancer, inflammatory and autoimmune disease and immune system development. A discussion of all of the functions attributed to NF-kB is beyond the scope of this chapter, and interested readers are directed to more in-depth reviews on the subject (Hayden and Ghosh 2012).

NF-kB binding activity is comprised of dimers derived from combinations of six different NF-kB proteins found in mammals that include RelA (p65), RelB, c-Rel, p100, p105 and Relish. RelA, RelB and c-Rel each have a transactivation domain within their C-terminus in addition to the Rel-homology domain that is shared with v-Rel, an oncoprotein expressed the avian reticuloentheliosis virus and all NF-kB family members. In contrast, p100 and p105 represent precursor molecules that must be processed into mature p50 and p52 binding subunits, respectively. Through interactions of their v-Rel homology domain (RHD), NF-kB family members have the capacity for form both heterodimers (e.g. p50/RelA) and homodimers (e.g. p50/p50) that affect transcription upon translocation and binding to conserved sequences within enhancers and promoters across a variety of genes.

The regulation of NF-kB activity within cells is complex and occurs primarily at a post-transcription and post-translational level allowing for quick activation of the pathway following appropriate signals. The primary mechanism of regulation occurs through binding of NF-κB dimers to inhibitor molecules known as inhibitor of kappa-B (IκB). IκB family members include the classical, cytoplasmic IκBα, IκBβ and IκBε molecules as well nuclear IκB molecules such as IκBξ and Bcl-3. The IκBs regulated NF-κB activity by binding dimers and sequestering these nuclear factors within the cytoplasm (classical IκBs) or limiting their binding activity within the nucleus (nuclear IkBs). Activation of the NF-κB in that is constrained within the cytoplasm is accomplished by signal-induced degradation of the IκB proteins leading to release of NF-κB dimers for translocation into the nucleus. IκB degradation is controlled by polyubiquitination following by targeting for proteolysis by the proteasome. The ubiquitination of IκB on Lysine residue 48 is in turn regulated by IκB phosphorylation via a kinase complex that involve a dimer of two catalytic subunits, IKKα and IKKβ along with a regulatory subunit termed NEMO (also known as IKKγ). Activation of NF-κB via

pathways such as via tumor necrosis factor receptor (TNFR) family receptors or the TLRs is accomplished through signal-regulated activity of the IKK complex. This regulation is controlled by interactions of NEMO with TNF receptor-associated factors (TRAFs) that form oligomeric assemblies at the membrane. These assemblies, nucleated by the cytoplasmic domains of TNFRs and TLR/IL-1β receptors, have been termed a signalosome that shows many similarities to the inflammasome that has already discussed. The interactions required for assembly of the TNFR- or TLR-associated signalosome again requires polyubiquitination reactions. The ubiquitination of TRAFs and additional proteins such as the TGF-β associated kinase (TAK1) that are also recruited to the signalosome lead to recruitment of the IKK complex via NEMO, and ultimately activation of IKK complex-mediated IκB phosphorylation, triggering IκB degradation and release of functional NF-κB dimers. This pathway of activation has been termed the canonical pathway of NF-κB activation. Alternative pathways of NF-κB activation that do not involve NEMO have been described, but their discussion is beyond the scope of this chapter. A more detailed description of the diversity of NF-κB signals and molecular mechanisms of activation can be found in focused review articles such as that written by Napetschnig and Wu (2013) or Hoesel and Schmid (2013).

3.1.2 Adaptive Immune Recognition

3.1.2.1 T Cell Receptor (TCR)

The alpha and beta chains of the TCR, which undergo marked sequence diversity during T cell development, confer the antigenic specificity for T cells. Upon ligation with antigenic peptide presented by major histocompatibility complex molecules, the TCR delivers the signals that trigger cytotoxic activity and cytokine production by T cells as well as their clonal proliferation necessary for a robust immune response.

The TCR alpha and beta chains have no inherent signaling functions by themselves. Instead, the signals that activate the T cell are derived from the associated chains of the CD3 molecular complex that includes 6 separate polypeptides: two CD3ξ, two CD3ε, CD3γ and CD3δ. These chains assemble along with the TCR into a macromolecular complex expressed on the T cell surface that is collectively termed the TCR/CD3 complex. Signal transduction by the TCR/CD3 complex is initiated by tyrosine phosphorylation of a conserved sequence present within the cytoplasmic domains of the various CD3 proteins that has been termed an immunotyrosine-based activation motif (ITAM). The CD3ξ chain contains 3 ITAMs and the other chains contain a single ITAM per chain for a total of 10 separate ITAMs within the TCR/CD3 complex. Tyrosine phosphorylation of the ITAMs within the TCR/CD3 complex is accomplished by two src-family kinases, lck and fyn that are expressed in T cells. The precise mechanism by which TCR engagement leads to the ITAM phosphorylation by lck or fyn is not fully understood. A leading model posits that the kinetic segregation of the CD45 phosphatase, the most abun-

dant membrane protein in T cells, from the TCR/CD3 complex following receptor ligation allows for sustained ITAM phosphorylation.

Following ITAM phosphorylation, the kinase Zap70 as well as adaptor molecules LAT and SLP76 are recruited to the TCR/CD3 complex. The assembly of these molecules into a signaling complex at the membrane subsequently activates a number of different downstream signaling pathways that lead to cytotoxic granule release by cytotoxic T cells, transcription and secretion of cytokines and the growth and division of the T cells that allows for self-renewal of T cells that have specificity for the target antigens.

The diversity of signals that are generated by the TCR/CD3 complex is significant. One of the most well described pathways is initiated by the activation phospholipase-C-γ (PLC-γ). PLC-γ cleaves the phospholipid, 3,4,5-phosphatidylinositol into two critical signaling intermediates. The first, diacylglycerol (DAG), is a potent activator of protein kinase C (PKC). There are several different PKC molecules expressed by T cells including PKC-β, PKC-θ and PKC-ξ. These kinases have many roles within T cells including the regulation of cytoskeletal polarity that is necessary for the delivery of cytotoxic granules to the interface between a T cell and its antigen-expressing target peptide/MHC complex (termed the immune synapse). PKC-θ also plays an important role in the activation of NF-κB signaling that drives transcription of genes including cytokines. The second signaling intermediate liberated by PLC-γ is inositol triphosphate (IP_3), which diffuses from the plasma membrane upon its liberation from membrane phospholipid. IP_3 is a potent activator of calcium channels within the endoplasmic reticulum (ER), and it leads to a rapid release of calcium and rise in cytoplasmic calcium concentrations that are normally maintained at very low levels. Calcium has a number of signaling effects within the T cells. One of the most important is the activation of nuclear factor of activated T cells (NFAT). The transcription of IL-2, a cytokine that is critical for T cell proliferation, is regulated by NFAT, which is normally excluded from the nucleus by its basal phosphorylation in addition to NF-kB and the AP-1 (Fos/Jun) transcription factor activated by MAP kinase pathways. The translocation of NFAT depends upon the removal of an inhibitory phosphorylation, which normally targets NFAT nuclear export. Following the rapid release of calcium triggered by IP3, the calcium-dependent phosphatase, calcineurin, removes the regulatory phosphorylation on NFAT allowing for its sustained translocation to the nucleus. Due to the importance of NFAT in T cell activation and IL-2 production, calcineurin represents an important pharmacologic target for regulating T cell immunity. Two FDA approved immunosuppressive drugs, cyclosporine A and tacrolimus inhibit the calcineurin phosphatase, and these drugs form the basis for most immunosuppressive drug regimens currently in use for solid organ and bone marrow transplantation. Despite the importance of NFAT in T cell activation, NFAT and calcineurin are expressed in numerous other cells throughout the body, and this signaling pathway play important roles beyond T cell activation likely contributing to many of the toxic effects observed with these immunosuppressive drugs.

Another major pathway for T cell activation includes the PI-3-kinase pathway that activates AKT (also known as protein kinase B). One of the most important downstream signaling pathways AKT is the activation of the mammalian target of rapamycin (mTOR) complex within cells. mTOR was discovered through its role as the molecular target of the immunosuppressive drug rapamycin (also known as siroli-

mus). Activated by many growth factor receptors including the TCR, nutrients (e.g. amino acids) and cellular energy state (AMP: ATP ratio), mTOR controls a number of metabolic processes that are critical to cell growth and proliferation including ribosomal biosynthesis, macromolecular biosynthesis in relation to nutrient availability and regulation of energy metabolism (Li et al. 2014; Shimobayashi and Hall 2014). mTOR is present within cells in two distinct, functional complexes. The first, known as mTORC1, is composed of mTOR along with the proteins sec-13 protein 8 (mLST8) and the regulatory-associated protein of TOR, also known as raptor. The other complex, mTORC2 replaces raptor with a rapamycin-insensitive companion of mTOR known as rictor as well as stress-activated protein kinase-interacting protein 1 (mSIN1). Of the two complexes of mTOR, mTORC1 is primarily sensitive to sirolimus; however, mTORC2 activity decreases after prolonged exposure, presumably due to mTOR depletion. The integration of nutrient signals with the TCR signals represents a critical function for mTOR as T cells must balance their growth and proliferation with nutrient availability. While mTOR is present within all cells, the particular sensitivity of T cells to mTOR inhibition is likely a result of the need for rapid cell growth and proliferation for a robust cell-mediated immunity.

While TCR signaling is critical for T cells activation, a number of different ligand-receptor systems integrate with TCR signals to co-stimulate T activation, proliferation and differentiation. CD28 represents one of the best characterized of these co-stimulatory receptors. This molecule, which binds to the ligands CD80 and CD86 on antigen presenting cells (APCs), delivers signals through a number of signaling pathways including PI-3-kinase and NF-κB that integrate with TCR signaling and are essential to drive the clonal expansion and differentiation of naïve T cells. The tight regulation of CD28 ligands on APCs represents one of the critical checkpoints to preventing aberrant stimulation of T cells and autoimmunity (Chen and Flies 2013). CD28 signals are further regulated by a CD28-related molecule, cytotoxic T lymphocyte attenuator 4 (CTLA4). CTLA4 is absent from quiescent naïve T cells, but rapidly expressed on the surface of T cells after TCR signaling. CTLA4 binds to CD80 and CD86, which are the same ligands that bind and activate CD28; however, CTLA4 both competes with the CD28 receptor for its ligands as well as delivers negative signals to the T cell that antagonize the TCR and CD28 signals. The critical importance of this negative regulatory receptor is illustrated by the massive autoimmunity that develops in mice as a result of uncontrolled T cell activation and proliferation driven by CD28 costimulation (Tai et al. 2007). CD28 and CTLA-4 are part of a much larger family of related receptors that both positively (e.g. ICOS) and negatively (e.g. PD-1) regulate T cells, and these pathways have turned out to be very important pathways that can be manipulated for therapeutic purposes in both transplantation and cancer (Sharma and Allison 2015; Ford et al. 2014).

3.1.2.2 B Cell Receptor (BCR) and Antibodies

Many of the proximal signal transduction events activated by the TCR have very similar counterparts in BCR signaling. Like the TCR/CD3 complex, the BCR is an alternatively spliced form of IgM that retains a transmembrane domain to allow

expression on the plasma membrane of B cells, but lacks any inherent signaling capability. Instead, the surface immunoglobulin (sIg) associates with two molecules Ig-α (CD79a) and Ig-β (CD79b) that act similar to CD3 complex proteins to provide the signaling capabilities of the BCR. Each CD79 molecule contains an ITAM within its cytoplasmic domains. Phosphorylation of these ITAMs in B cells is accomplished by src-family kinases lck and fyn. The mechanisms of BCR signal initiation remain as elusive as that of the TCR. Binding and crosslinking of sIg by multivalent antigenic ligands is generally required for optimal BCR activation (Liu et al. 2010). Conformation changes within the complex and molecular segregation models similar to the TCR have been proposed, but ultimately, BCR engagement leads to stable ITAM phosphorylation of the CD79 complex molecules, and Syk, a kinase closely related to Zap70 is recruited. Syk recruitment and activation leads to triggering of PLC-γ and PI-3-Kinase signaling cascades that eventual activate a wide range of signaling pathways including NF-κB (Thome et al. 2010) and AKT/mTOR (Limon and Fruman 2012).

3.1.3 Immune Recognition that Blurs the Lines Between Innate and Adaptive Immunity

3.1.3.1 Fc Receptors

Antibodies produced by B cells carry out a range of effector functions including neutralization, opsonization and cytotoxicity that are critical to effective immunity. However, with the exception of neutralization, antibody effector function relies upon other elements of the immune system. The recognition that antigens bind to the surface of cells such as macrophages in the presence of specific antibody led to the discovery of specific receptors that bind to the constant domains of antibody found in the Fc portion of the antibody molecule. Over the course of the past half-century, Fc receptors (FcRs) that specifically bind IgG (FcγRI [CD64], FcγRII [CD32] and FcγRIII [CD16]), IgA (FcαRI [CD89]) and IgE (FcεRI) have been identified. The range of different receptors for antibody, their patterns of expression and their signaling give rise to a highly complex system that is central to both the effector function and regulation of adaptive immunity (Ravetch and Nimmerjahn 2013).

FcR expression is found across most cells of the myeloid (i.e. monocytes, macrophages, mast cells, neutrophils and eosinophils) and lymphoid lineage (i.e. B cells and NK cells). The canonical FcRs are all type I integral transmembrane proteins that belong to the immunoglobulin protein family. With the exception of FcγRII, activating FcRs have structural similarities to other immunoreceptors such as the TCR and BCR in which the Fc-binding domain (generally referred to as the α subunit) is expressed in a separate protein subunit from the signaling subunit (referred to as the γ subunit). The ITAM within the γ subunit plays a critical role in triggering the effector functions of antibodies in cells that express the specific Fc-binding α subunit. In the case of FcεRI and FcγRIII, an additional β subunit is also part of the receptor complex, which

also contains an ITAM within the cytoplasmic C-terminal domain of the protein. This latter subunit belongs to a family of proteins with 4 membrane-spanning regions that is related to CD20. In contrast to the other activating FcRs, FcγRII is encoded as a single polypeptide chain encoded by two genes in humans. FcγRIIA, which is absent from all mammals other than primates, encodes a protein with an Fc binding domain in the N-terminus and an ITAM within the cytoplasmic C-terminus similar to the γ subunit of the other activating FcRs (Hogarth 2002). FcγRIIB, which is present in all mammals, exists as two separate splice forms. Both splice forms contain an ITIM within its cytoplasmic domain, and this receptor, unlike its activating counterpart, plays an important regulatory role by setting the threshold for activation of effector cells as well as repressing B cell activation (McGaha et al. 2005). Additionally, non-canonical FcRs have also been described that belong to distinct protein families that include the MHC class I-like neonatal FcR (FcRn) (Stapleton et al. 2015) and the IgM-binding FcμR (Wang et al. 2016). FcRn has been shown to play many roles, most notably in antibody transport, and this unique FcR appears critical for the distribution and clearance of circulating serum antibody (Stapleton et al. 2015).

Studies from knock out mice have demonstrated that the range of effector responses following FcR engagement by antibody is highly dependent upon the balance between both activating and inhibitory FcRs (Takai et al. 1994; Boruchov et al. 2005; Clynes et al. 1999). While expression of individual FcRs contribute to the balance in response, the functional binding of antibody to different FcRs also influences the response. Binding affinity differs considerably across the different FcRs for IgG with FcγRI having the highest affinity and FcγRIII having the lowest relative affinity. However, these binding affinities are also highly dependent upon the isotype. Some IgG isotypes such as human IgG1 show much higher binding affinity for FcγRI compared with the low affinity of human IgG4 or absence of appreciable binding to IgG2 isotypes (Daeron 2014). FcRs also influence the signals from other immunoreceptors such as the BCR where co-ligation of FcgRIIB by immune complexes on follicular dendritic cells leads to inhibition of B cell activation and proliferation (Muta et al. 1994; Tzeng et al. 2005). A thorough review of FcR structure and signaling can be found in several recent and thorough reviews (Daeron 2014; Bruhns and Jonsson 2015).

3.1.3.2 NK Cell-Associated Receptors

The immune system has devised a plethora of ways to recognize pathogens. The NK cell presents one of the most illustrative examples of this diversity. As sentinels of the innate immune system that search for and destroy compromised host cells such as virally infected cells, NK cells have acquired a range of activating and inhibitory receptors that provide some specificity for recognition of altered self, yet tightly regulate the potent cytotoxic activity of these cells (Pegram et al. 2011). Keeping in line with their function, NK cell-associated receptors can generally be grouped into activating and inhibitory receptors. While this classification approach is convenient, it is important to recognize that some NK receptors such as the SLAM family of receptors can deliver both activating and inhibitory signals that depend upon their

context and the co-expression of cytoplasmic adaptor molecules (Cannons et al. 2011). In addition, NK cell-associated receptors have also undergone rapid evolution giving rise to marked divergence in these receptors across species.

3.1.3.3 KIR and Ly-49 Families of Receptors

The killer immunoglobulin-like receptor (KIR) and Ly49 receptor families share largely overlapping functions. Both families provide activating and inhibitory receptors that regulate the cytotoxic activity of NKs. The inhibitory members of both families utilize the familiar ITIM found in other inhibitory immunoreceptors such as FcγRIIB and PD-1. The activating family of receptors lack inherent signaling, but instead acquire their signaling through interactions with the ITAM-containing adaptor molecule, DNAX associated protein of 12 Kd (DAP12, also known as KARAP12). Finally, both families encode for proteins with an extracellular domain that bind to MHC class I or MHC class I-like molecules. Despite these tremendous similarities, KIRs are type I integral transmembrane proteins that are members of the immunoglobulin gene superfamily, and the KIR gene family is only present within primates. In contrast, Ly49 receptors are type II integral single-pass transmembrane proteins that are members of the C-type lectin family. This family of receptors is found in rodents, but absent from primates. The significant functional similarities combined with the marked structural differences suggest that these receptors convergently evolved to perform a critical function in NK cells and perhaps in T cells, which also express these receptors in some subsets (Rahim and Makrigiannis 2015; Thielens et al. 2012; Campbell and Purdy 2011).

The KIR family of receptors is highly polymorphic. Eight different inhibitory KIRs have been described. All inhibitory KIRs contain a long cytoplasmic domain containing one or two ITIMs, but two principle structural groups exist. One group comprised of receptors with two extracellular immunoglobulin (Ig) domains, which are referred to by the use of "2D" along with "L" in the name to denote the long cytoplasmic domain, include: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4 and KIR2DL5. Three additional KIRs containing three extracellular Ig domains have also been described that include KIR3DL1, KIR3KL2 and KIR3DL3. In contrast, the activating KIRs are comprised of five receptors with two Ig domains (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4 and KIR2DS5) and one with three Ig domains (KIR3DS1). The activating receptors have highly similar ectodomains, but a short cytoplasmic domain with the use of "S" in the nomenclature to indicate this form of the KIR. The short cytoplasmic domain lacks any known signaling motifs, and as discussed, these receptors gain their signaling capacity through interactions with DAP12 or other ITAM-containing adaptor molecules through interactions between charged amino acid residues within the transmembrane domains of the proteins in a similar fashion to other activating immunoreceptors (Call and Wucherpfennig 2007). Within each of these KIR genes, multiple variant alleles have been identified through population studies in humans with KIR3DL3 having more than 100 known allelic variants. Given the potential importance of

these polymorphisms to human disease, efforts to catalogue the wide range of KIR polymorphisms are currently underway by the Immuno Polymophism Database (IPD) and IMGT/HLA database (Robinson et al. 2015).

HLA-C forms the primary ligand for inhibitory KIR receptors with two Ig domains. In contrast, the three Ig domain receptors appear to bind HLA-A and HLA-B molecules. KIR3DL1 appears to preferentially bind HLA-B alleles of the Bw4 serologic group whereas KIR3DL2 shows higher affinity for HLA-A3 and HLA-A11 molecules. Crystal structures of inhibitory KIRs indicate that the binding to MHC class I molecules straddles the peptide binding cleft suggesting that the bound peptide may influence the affinity of the KIR-MHC interaction. Nevertheless, inhibitory KIR binding to MHC appears far more promiscuous than the binding of TCR to peptide-bound MHC (Campbell and Purdy 2011). While the ligands for the inhibitory KIRs have begun to be elucidated, the ligands for activating KIRs are far less well understood. KIR3DS1, KIR2DS2 and KIR2DS1 are predicted to have specificity for HLABbw4, HLA-C1 and HLA-C2, respectively. While in vitro binding data supports the KIR2DS1 and KIR3DS1 (Stewart et al. 2005; O'Connor et al. 2015), KIR2DS2 has structural data to support a role for HLA-A*11:01 as a ligand (Liu et al. 2014).

Analogous to the KIR system in primates, the Ly49 receptors also recognize MHC class I molecules and are highly polymorphic. Ly49 genes are found across mammals including the presence of a single, non-functional gene in humans; however, Ly49 locus has undergone significant gene amplification in rodents and to a lesser extent in horses analogous to the amplification of the KIR locus in primates. The highly polymorphic nature of Ly49 and KIR genes is likely directly related to the highly polymorphic nature of its MHC ligands, which represent the most polymorphic genes in mammals (Rahim and Makrigiannis 2015).

The mechanism by which activating and inhibitory KIR and Ly49 molecules regulate NK cell function still remains poorly understood. Early models suggested that NKs utilize the recognition of self-MHC by inhibitory receptors as a tolerance mechanism to block cytotoxicity against self by overriding all but the most potent activating signals. While this early "missing self" hypothesis may be operative in some settings, it fails to explain the absence of NK auto-reactivity in the setting of MHC class I deficiency. In fact, NK cells isolated from MHC class I deficient mice display defective cytotoxicity. These observations have led to the concept of "licensing" in which the interactions between self-MHC and inhibitory KIR or Ly49 molecules are required during NK cell development to ensure that only NK cells with receptors for self-MHC are functional (Elliott and Yokoyama 2011).

3.1.3.4 CD94:NKG2A/C Receptors

The CD94:NKG2A and CD94:NKG2C receptors, like KIR and Ly49, interact with MHC class I molecules. These C-type lectin-like receptors serve as an inhibitory receptors (CD94:NKG2A) or activating receptors (CD94:NKG2C) that associate with the familiar DAP12 molecule to provide important signals to NK cells (Lanier et al. 1998). In contrast to the KIR and Ly49, CD94:NKG2A/C receptors exhibit much less genetic diversity in the population. This reduced diversity likely relates to

the non-polymorphic nature of their ligand, HLA-E (Braud et al. 1998; Brooks et al. 1997). This non-classical MHC class Ib molecule appears to serve as a molecular sensor of MHC expression by cells by presenting leader peptides derived from the classical MHC class I molecules (Braud et al. 1997). The Qa-1 molecule provides a similar function in mice (Vance et al. 1999). In the context of some viral infections such as the herpesviruses, the reduction in MHC class I expression by the virus also reduced the expression HLA-E. This reduction in HLA-E expression appears to relieve the inhibition enforced by CD94:NKG2A to allow for NK cell activation and function. CD94:NKG2C, which is an activating receptor for NK cells also binds to HLA-E; however, this binding is much lower in affinity compared to NKG2A. NKG2C, but not NKG2A, has also been shown to bind to virally encoded MHC class I homologs such as UL18 supporting the existence of important differences in specificity that contribute to their functional roles (Kaiser et al. 2008).

3.1.3.5 NKG2D: A Receptor for Altered Self

Unlike related NKG2 family members, NKG2D is a type II integral transmembrane protein of the C-type lectin family that does not associate with CD94. It exists as two splice forms in mice. The longer splice variant associates with a second transmembrane protein known as DAP10 that contains a conserved YXXM motif similar to that found in costimulatory molecules of the CD28 family (Chang et al. 1999). The shorter splice variant of NKG2D associates with DAP10 along with DAP12 that contains a canonical ITAM in its cytoplasmic domain (Diefenbach et al. 2002). Engagement of NKG2D by its ligands is a potent activator of cytotoxicity as well as cytokine secretion by NK cells. NKG2D is also expressed by NK-T cells and subsets of α/β- and γ/δ-T cells. Interestingly, humans only expressed the long form of NKG2D that associates with DAP10, but this receptor appears capable of triggering cytotoxicity despite the absence of a known ITAM-containing receptor partner (Wu et al. 1999).

A number of ligands have been identified for NKG2D. The best-characterized NKG2D ligands in humans belong to the MHC class I polypeptide-related sequence (MIC) or UL16 binding protein (ULBP) genes (Bauer et al. 1999; Cosman et al. 2001). Both genes encode for proteins with sequence and domain structure that is related to MHC class I proteins; however, β2-microglobulin is not required for surface membrane expression (Li et al. 2001). DNA damage or heat shock-induced cellular stresses induce MIC-A, MIC-B, and ULBP1-4 genes (Groh et al. 1996). While the ligands for NKG2D in mice are also highly homologous to MHC class I, the stress-induced retinoic acid early transcript 1 (Rae-1) molecules form the major ligands along with ULBPs and histocompatibility 60 (H60) proteins (Cerwenka et al. 2000; Carayannopoulos et al. 2002; O'Callaghan et al. 2001). Adding to the complexity of studies of NKG2D, the ligands for this important activating receptor differ across mouse strains. These differences can lead to outcomes that differ based upon the genetic background and associated ligands (Ogasawara et al. 2005). Nevertheless, deficiency of NKG2D is not associated with any numerical changes in the numbers of NK cells or T cells. NKG2D-deficient mice do exhibit an increased

frequency of tumors as well as impaired anti-tumor immunity suggesting that this pathway may play an important role in tumor surveillance (Guerra et al. 2008).

3.1.3.6 Natural Cytotoxicity Receptors (NCRs)

Identified in the 1990s by the ability to activate NK cells, the NCRs that include NCR1 (NKp46), NCR2 (NKp44) and NCR3 (NKp30) show little sequence or structural similarities other than being members of the Ig superfamily. All NCRs are type I integral transmembrane proteins that interact with homodimers of DAP12 or heterodimers of the γ-chain of the FcR (FcRγ) or CD3ξ to generate activating signals for NK cells. While NCRs are expressed by all NK cells, they are also expressed by other cell types including ILCs and some T cell subsets. Although the original description of these receptors demonstrated an association with tumor cell lysis by NK cells, these receptors appear to bind to a broad array of ligands derived from viruses, bacteria, parasites and tumor cells. The structural mechanisms by which these receptors bind such diverse ligands is not well understood (Kruse et al. 2014).

3.1.4 Immune Effector Molecules

3.1.4.1 Defensins and Other Antimicrobial Peptides

Species from invertebrates to humans produce a diverse group of antimicrobial peptides (AMPs, also called host defense peptides [HDPs]) that play important roles in innate host defense (Mansour et al. 2014). In humans, AMPs include anionic peptides such as dermcidin peptide, DCD-1L produced by eccrine sweat glands (Paulmann et al. 2012) as well as an array of cationic peptides. These latter cationic AMPs include linear cationic (also known as α-helical) peptides such as the cathelicidin, LL-37, the only known cathelicidin in humans (Nijnik and Hancock 2009; Zhang et al. 2015), and cysteine-rich, cationic peptides of the defensin family.

Defensins are the most diverse AMPs known in humans. These peptides exhibit broad antimicrobial activity against enveloped viruses, fungi and bacteria. Originally identified as a component of the azurophilic granules of neutrophils in humans and rabbit, defensin expression has also been observed in a wide range of epithelial cells including tracheal, bronchial, vaginal and mammary epithelium. Structurally, defensins exhibit high sequence variation with three pairs of conserved cysteine residues that produce intra-molecular disulfide bonds defining this class of AMPs. Three different classes of defensin molecules have been identified in mammals, termed α, β and θ. Neutrophils produce primary α-defensins whereas β-defensins are the primary defensins produced by epithelial cells. There are six θ-defensin genes in the human genome. All of these genes appear to contain a premature stop codon that precludes translation of θ-defensin peptides; however, at least one primate θ-defensin peptide has been isolated from rhesus macaques.

AMPs appear to mediate their antimicrobial activity through a number of mechanisms. The most intensely studied AMPs are the cationic peptides, especially the linear cationic peptides. These positively charged peptides appear to interact with the negatively charged lipids of cell membranes leading to membrane disruption and cell lysis (Brogden 2005). Although direct antimicrobial mechanisms are an important part of the mechanism of action for AMPs, linear cationic peptides and defensins have also been shown to play a role in the regulation of immunity. The cathelicidin, LL-37 acts as a chemotactic agent for mast cells and monocytes, modulates TLR signaling and also appears to be a potent inducer of the inflammasome (described in more detail below) (Kahlenberg and Kaplan 2013). β-defensins have also been shown to attract dendritic cells and T cells to sites of inflammation through binding to the chemokine receptor, CCR6 (Yang et al. 1999). In addition, the murine β-defensin, mBD-14 appears to also play a role in the induction of regulatory T cells indicating that the role of AMPs in host defense are complex (Navid et al. 2012).

Innate immunity begins at epithelial surfaces, which provide an anatomic barrier to limit exposure to pathogens. Epithelial surfaces have acquired a number of physiologic mechanisms that further counteract pathogen entry including desquamation to shed pathogens adherent to epithelium, secretions that constrain pathogen mobility (e.g. mucous and saliva) and viability (e.g. acids, lysozyme and defensins) and motility mechanisms (e.g. peristalsis and cilia) to promote clearance from the surface. It is also becoming increasingly apparent that non-pathogenic microorganisms that comprise the so called "microbiome" that inhabit all epithelial surfaces provide additional defense by competing with pathogens in a largely symbiotic relationship (Kamada et al. 2013). Although our understanding of the role of the microbiome in immunopathology is only in its infancy, recent studies have shown significant changes in the microbiome associated with a number of inflammatory conditions including inflammatory bowel disease (Frank et al. 2011), rheumatologic disease and (Volkmann et al. 2016) and skin disease (Zeeuwen et al. 2013).

Once an organism breaches the epithelial surfaces, there are a number of innate immune mechanisms that recognize and respond to the pathogenic threat. One of the most well-studies mechanisms involve soluble molecules such as mannose binding lectin (MBL) that recognizes abnormal pathogen-related patterns of glycosylation from normal patterns of glycosylation. Upon ligand binding, MBL is a potent activator of the complement system.

3.1.4.2 Complement

Originally recognized for its roles in innate immunity, the complement system is increasingly recognized as playing important roles as effector molecules acting directly on pathogens as well as helping to shape the nature of the innate and adaptive immune response. Over 30 different molecules, soluble or surface-expressed, contribute to the complement system. In the classical pathway of complement activation as shown in Fig. 3.1, a hexameric complex referred to as C1 comprised of three subunits termed C1q, C1r and C1s binds to Fc regions of antibodies. This

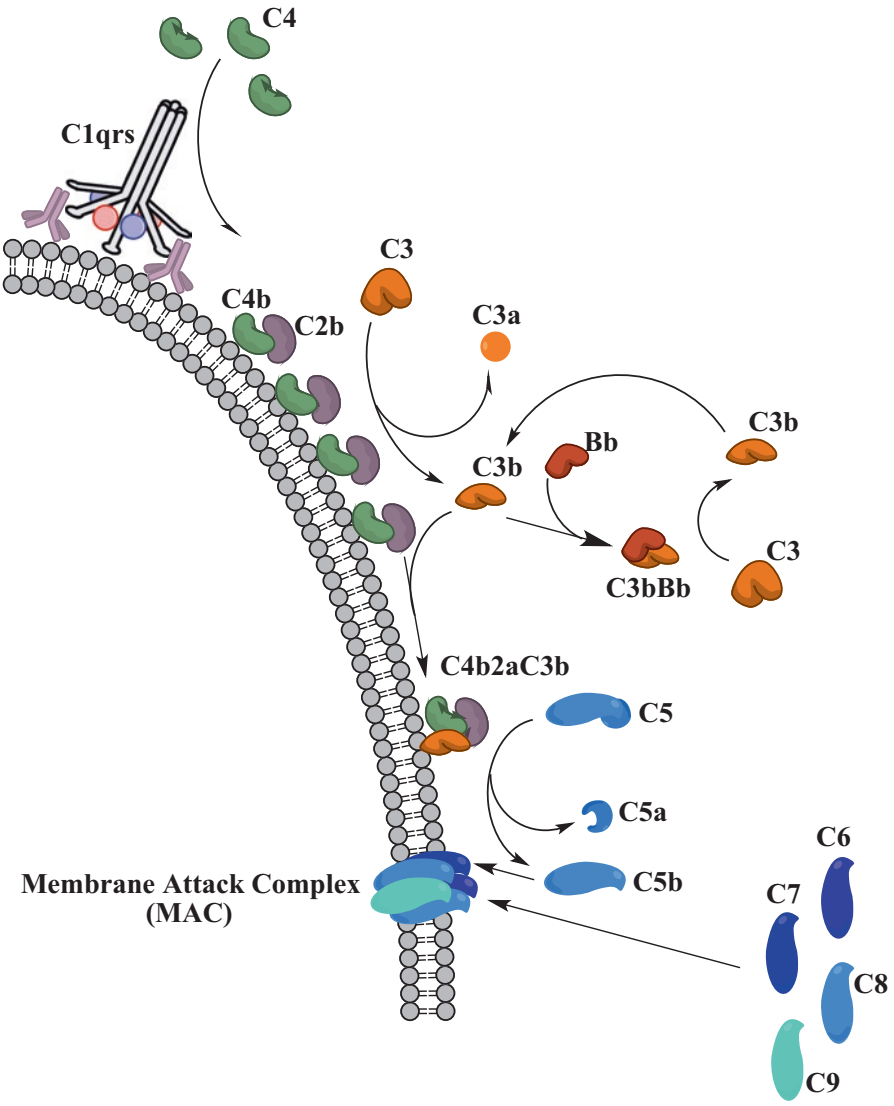


Fig. 3.1 In the classical pathway of complement activation, a complex composed of three subunits (C1q, C1r, and C1s) of C1 bind to the Fc regions of antibodies. A resultant conformational change in C1s activates its enzymatic activity, which cleaves C2 into C2a and C2b, and C4 into C4a and C4B. C4b binds to cell surfaces, and this binding recruits C2b to form a new enzyme ('C3 convertase') that cleaves C3 into C3a and C3b. C3b complexes with an additional protein known as B, which is subsequently cleaved to yield Bb. The C3bBb complex is also a C3 convertase that can tremendously amplifiy the production of C3b. The C3b binds to the cell surface and produces a complex with C2bC4b (C5 convertase) that cleaves C5 into C5a and C5b, resulting in the assembly of the terminal C6 to C9 components into a pore-forming complex ("membrane attack complex, MAC") that causes cell lysis

binding induces a conformational change in C1s that activates its enzymatic activity. Catalytically active C1s cleaves two other complement proteins, C4 producing C4a and C4b and C2 producing C2a and C2b. The C4b produced by this reaction binds covalently to cell surfaces through a thio-ester bond, and this binding subsequently recruits C2b to form a new holoenzyme with catalytic activity towards C3 resulting in the producing of C3a and C3b, hence this complex is called a "C3 convertase". The liberated C3b then complexes with another factor called B that is subsequently cleaved to Bb. This C3bBb complex is also a C3 convertase capable of tremendously amplifying the producing of C3b. The large amount of C3b, like C4b, also binds to the cell surface through thioester bonds producing a complex with C2bC4b that is capable of activating C5 (hence termed a "C5 convertase") into C5a and C5b that is capable of activating the assembly of the terminal C6 to C9 components of the complement system into a pore-forming complex on the cell surface, termed the "membrane attack complex" or MAC, that leads to lysis of the cell (reviewed in (Mathern and Heeger 2015)). In addition to direct cell lysis, the complement deposited onto the surface of a cell or antigen-antibody complex is also recognized by complement receptors on B cells and myeloid cells that lead phagocytosis (a process termed opsonization) (Holers 2014). In addition to triggering opsonization and effector function, complement receptors also activate these cells leading regulation of antibody production (Pepys 1974; Dempsey et al. 1996). The C3a and C5a products of complement activation also play roles in bridging innate and adaptive immunity. These molecules, frequently termed "anaphylatoxins" induce both local changes in the tissue microenvironment such as vasodilation as well as serving as important chemotactic signals for cells including neutrophils and monocytes (Guo and Ward 2005; Klos et al. 2009). In addition to providing potent activating signals to a range of antigen presenting cells that enhance the processing and presentation of antigen, C3a and C5a receptors are also expressed by T cells providing important costimulatory signals for these cells (Strainic et al. 2008).

While an antibody-driven mechanism was the first recognized pathway for the activation of the complement enzymatic cascade, several alternate pathways have been described. Innate recognition molecules such as MBL already described provide one important activation pathway; however, the complement system appears to also be quite poised for action without the need for additional activator molecules. Specifically, C3 is capable of spontaneous hydrolysis to form what has been termed C3(H_20). This alternate form of C3 is capable of recruiting factor B and factor D. The factor D, upon recruitment, cleaves factor B to form Bb which subsequently is able to enzymatically cleave C3 to C3b thereby forming the C3 convertase independently of C2bC4b. The membrane recruitment and stability of the C3bBb complex is further potentiated by another protein called properdin, which directly binds to microorganisms like bacteria (Spitzer et al. 2007; Fishelson et al. 1984).

The importance of C3 convertase as a central point for both amplification and downstream activation of the terminal pathways of complement makes this complex an important site for regulation. Several membrane proteins play a large role in restraining the activation of this potent effector system on normal host cells. The first is decay accelerating factor (also known as DAF or CD55). DAF is a

GPI-linked protein facilitates the dissociation of Bb from the C3bBb complex as well as C2b from the C2bC4b complex thereby reducing the level of C3 convertase as well as C5 convertase activity (Fujita et al. 1987). CD46 (also known as membrane cofactor protein [MCP]) is another surface-expressed enzyme that cleaves C3b to an inactive form (iC3b) counteracting the amplification of C3 activation that is critical to the complement pathway (Cole et al. 1985; Kim et al. 1995). C3 activation is further constrained by factor H, a soluble protein that binds to glycosaminoglycans within the extracellular matrix and surface-deposited C3 leading to both dissociation of Bb from the C3 convertase complex as well as inactivation of C3b to iC3b (Makou et al. 2013). While C3 convertase activity is a principle point of complement control, CD59, another membrane protein found ubiquitously on host cell membranes, primarily regulates MAC formation by binding C8 and blocking C9 incorporation that is essential for formation of a functional MAC (Morgan et al. 2005).

3.1.4.3 Cytokines

The term "cytokine" generally refers to an array of soluble mediators secreted by cells. These molecules perform both effector and regulatory functions within the immune system. They act primarily locally either on the cell producing them such as the autocrine effect of IL-2 to promote T cell growth and proliferation or on surrounding cells such as the paracrine effect of T cell-derived IL-4 on B cells to promote isotype switching. However, many cytokines can also act systemically in an endocrine-like fashion such as the cytokines IL-1 and IL-6 that contribute to the febrile response through their actions on the hypothalamus. Thus, the distinction between a cytokine, a growth factor and a hormone is somewhat arbitrary. Nevertheless, the term cytokine is usually reserved for those soluble mediators involved in immunity whereas growth factors at more on somatic cells for normal organismal physiology.

Classifying cytokines is challenging. Early classification schemes were based upon the cell types producing the cytokines leading to terms "monokines" for those produced by monocytes and "lymphokines" for those produced by lymphocytes. However, these terms pose significant problems due to the non-absolute nature of many cytokines in their cell of origin. The role of cytokines in immunity underlies the commonly agreed upon nomenclature of "interleukin". This nomenclature, while somewhat of misnomer as many cytokines are produced by cells outside of the immune system such as keratinocytes, provides a common language to at least discuss these important molecules in immunity. Given the relatively recent molecular characterization of cytokines and their receptors, these molecules are probably best classified by their structural and receptor signaling similarities into four principle families as shown in Table 3.1. Despite the shared structure and receptor signaling, the functions of cytokines within each family are highly diverse. Despite more than a half-century of study into the interferon family of cytokines, which were first recognized as important effector molecules in antiviral defense, we are

still discovering new roles for these molecules in regulation of immunity (Chow and Gale 2015). The reader is referred to textbooks of immunology for more in-depth discussion of the many cytokines and their role within the immune system and beyond.

Type I Cytokines

The type I cytokines can be grouped together based upon a shared molecular structure that is comprised of four α-helical domains bundled together as shown in Fig. 3.2. Comparison of these helical domains show that there are two conserved families in which the α-helices are either approximately 15 (short-domain) or 25 (long-domain) amino acids in length. The short α-helical domain cytokines include interleukin (IL)-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-15, IL-21 and TSLP. The long α-helical cytokines include IL-6, IL-11, IL-27, leukemia inhibitory factor (LIF), oncostatin-M (OSM), growth hormone (GH) and erythropoietin (Epo). The structural similarities of type I cytokines extend to the receptors used by these molecules. Both long and short domain molecules utilize receptors with a conserved motif in their extracellular domain along with conserved cysteine residues involved in intra-molecular disulfide bonding necessary to generate a receptor capable of binding the type I cytokines.

The diversity of signaling induced by type I cytokines is conferred by receptor diversity for each cytokine. Many of the type I cytokines involved in immunity share subunits leading to an additional, alternate approach to classifying these cytokines. A group of the short helical domain cytokines (IL-2, IL-4, IL-7, IL-9, IL15 and IL-21) use a common receptor subunit termed the common γ-chain (γ_c) for their signaling. The severe combined immunodeficiency (SCID) associated with X-linked genetic defects of the γ_c highlight the importance of these cytokines to both adaptive and innate immunity. A second group of short helical domain cytokines comprised of IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) utilize an alternate common subunit that is referred to as common β-chain (β_c). The

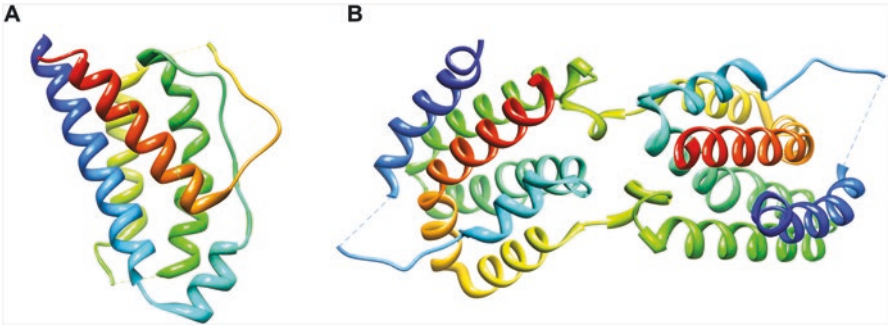


Fig. 3.2 **a**. Ribbon diagram of the type I cytokine, IL-2, showing the four conserved alpha-helical domains. **b**. Ribbon diagram of the type II cytokine, IL-10, showing the homodimeric structure with 6 alpha-helical domains in each dimer

long helical domain cytokines that include IL-6, IL-11, OSM, LIF and IL-27 utilize a common receptor subunit known as gp130. In all cases, the common receptor subunit requires dimerization with additional subunits to form functional receptors for the individual cytokines. The complexity is increased even further with some cytokines even sharing the γ_c and β-chain, and instead use a third subunit to confer cytokine specificity to the receptor complex as illustrated by IL-2 and IL-15. Both use a heterodimer of the IL-2Rβ chain with the γ_c; however, IL-15 signaling through this complex requires its binding and presentation by the IL-15Rα chain in a process that has been termed trans-presentation (Dubois et al. 2002). This molecular complexity of the receptor system for these cytokines can give rise to quite different phenotypes of mice bearing genetic deletion of the different subunits. Whereas mice lacking the γ_c show severe immunodeficiency due to the failure in development of T cells and B cells primarily due to the deficiency in IL-7 receptor signaling, deletion of the IL-2Rβ or IL-2Rα subunits leads to severe autoimmunity that arises from an absence of regulatory T cells that require IL-2 for their maintenance (Malek and Castro 2010).

Interferons and the IL-10 Family of Cytokines

The interferon (IFN) and IL-10 family of cytokines share significant structural similarity that also extends to their receptors, which has many parallels to the type I cytokines. As shown in Fig. 3.2, the type II cytokines possess a conserved structure with five to six alpha-helical domains that exist in a biologically active form as homodimers. The IFN family, which hold the distinction of being the first cytokines discovered in the 1950s, were originally identified by their ability to induce an antiviral state within cells. Over the past century, the family of pleiotropic cytokines have been shown to exhibit a wide range of functions within the innate and adaptive immune system that contribute to host defense and disease in complex ways.

The IFNs can be divided into three distinct groups that are termed type I, type II and type III IFNs. This classification should not be confused with the same nomenclature used for cytokines more broadly. Type I IFN consists of IFN-α (formerly called leukocyte IFN), IFN-β (formerly called leukocyte IFN), IFN-ω, IFN-τ along with a few additional subtypes. IFN-α is not a single molecule, but instead encoded by 12 different genes in mice and humans. A single gene encodes IFN-β. The production of type I IFN is regulated by several signaling pathways that include the TLRs and nucleic acid sensing receptors such as RIG-I that were previously discussed in the section on innate immune recognition. While many different cells are capable of producing type I IFNs, IFN-α is primarily produced by myeloid cells, especially the specialized dendritic cells (DCs) known as plasmacytoid DCs that produce large quantities of type I IFN following TLR activation.

In addition to sharing significant sequence similarity, IFN-α and IFN-β also share a common receptor that is referred to as the IFN-α/βR (or Type I IFNR). The ability of type I IFNs to induce an antiviral state within cells depends upon the expression of a number of genes such as 2,5-oligoadenylate synthetase (OAS),

myxovirus resistance gene A (MxA) and the double stranded RNA-responsive protein kinase R (PKR) that interfere with viral replication and spread (Sadler and Williams 2008). While the type I IFNs, through induction of this antiviral state are important contributors to innate immunity, type I IFNs also play significant roles in adaptive immunity. IFN-α can induce the differentiation and maturation of DCs to enhance antigen presentation. Type I IFNs are also capable of promoting T cell survival and modulating effector T cell differentiation to enhance type I polarization of immunity (i.e. Th1 in CD4+ T cells and cytotoxic CD8+ T cells) and T cell memory (Tough 2012). The reasons for the marked diversity in type I IFNs is unknown; however, it is apparent that individual subtypes of the type I IFNs possess different biologic activities suggesting that the type I IFN system is more complex than currently appreciated (Pestka et al. 2004).

Unlike the diversity in type I IFNs, type II IFNs consist of a single molecule, IFN-γ, which utilizes its own receptor (known as the IFNγR or type II IFNR) that is distinct from the type I IFNR. IFN-γ is an important immune-stimulatory and pro-inflammatory cytokine produced by T cells, primarily CD4+ Th1 cells and CD8+ cytotoxic T cells, NK cells and NK-T cells. IFN-γ is essential for control of intracellular pathogens. Disruption of IFN-γ or its receptor leads to increased susceptibility to viruses, parasites such as *Toxoplasma gondii* and bacteria including *Salmonella* sp. and mycobacteria (John et al. 2002; Dalton et al. 1993; Huang et al. 1993). It also plays role in anti-tumor immunity (Rosenzweig and Holland 2005; Kaplan et al. 1998). The mechanisms by which IFN-γ modulates immunity are manifold. It is a potent activator of monocytes and macrophages, inducing cytokines and antimicrobial pathways such as oxygen radicals that lead to killing of intracellular pathogens. IFN-γ is a potent inducer of antigen presentation through the up-regulation of major histocompatibility complex (MHC) proteins, TAP1/2 peptide transporters and the proteasome that are all essential for the robust processing of intracellular proteins into peptides for class I MHC presentation (Fruh and Yang 1999). IFN-γ is also an important regulatory cytokine, inhibiting the generation of Th2 and Th17 differentiated CD4+ T cells. Given the potent effects of IFN-γ, it is also subject to tight regulation within a complex cytokine network. Production of IFN-γ by T cells is enhanced by IL-12, IL-18 and IL-27 whereas tumor growth factor-β and IL-6 suppress its production (Schoenborn and Wilson 2007).

Type III IFNs are comprised of the IFN-λ molecules (also known as IL-29 [IFN-λ1], IL-28A [IFN-λ2], IL28B [IFN-λ3] and IFN-λ4 in humans), which show sequence and functional similarity to type I IFNs (Sheppard et al. 2003; Kotenko et al. 2003); however, these molecules bind to a distinct heterodimeric receptor that shares the β-subunit with IL-10 (Miknis et al. 2010). Myeloid lineage cells and epithelial cells are the primary sources of IFN-λ with the signals inducing its expression largely overlapping those inducing type I IFNs. The effects of IFN-λ are primarily focused on epithelial cells due to preferential expression of the IFN-λ receptor complex, and IFN-λ plays important roles in immune responses at epithelial surfaces. High levels of expression are observed in respiratory and gastrointestinal epithelial cells with increases susceptibility to a variety of viral pathogens in mice lacking the α-subunit of the IFN-λ receptor. It also appears to be important for

viral hepatitis with genetic polymorphisms within the IFN-λ locus associated with improved outcome from hepatitis B and hepatitis C viral infection. More recently, IFN-λ has also been implicated in regulation of the blood-brain barrier (BBB) with mice lacking the IFN-λ receptor showing increased BBB permeability and increased titers of West Nile virus in the central nervous system following infection. IFN-λ also appears to play roles in regulating adaptive immunity with effects on T cells and the leukocyte trafficking. While type I and type III IFNs largely appear redundant in function, the tissue compartmentalization of these cytokines with type III IFNs restricted in expression and function mostly to epithelial surfaces may be an important means for controlling immune responses at these environmental barriers without routinely activating systemic inflammation. Many of the similarities and differences between type I and type III IFNs can be found in recent reviews such as Lazear et al. (2015). Given the relatively recent discovery of IFN-λ in 2003 compared to the IFN family as a whole that was described over 60 years ago, it is likely that many unique functions of type III IFNs will be identified in the future.

Although the type III IFNs show sequence similarity with type I IFNs, they show greater structural similarity to the other type II cytokines of the IL-10 family. Originally identified as a Th2-derived cytokine that inhibited cytokine synthesis, IL-10 is produced by many different immune cells including most CD4+ T cell subsets including regulatory T cells, CD8+ T cells, NK cells, B cells, myeloid lineage cells such as macrophages and DCs and tissue-resident mast cells. Many of the effects of IL-10, mediated through its unique receptor, serve to dampen inflammatory responses. Some of these effects include induction of suppressor of cytokine signaling (SOCS) molecules that limit cytokine receptor signaling, direct inhibition of cytokine gene expression and inhibition of MHC class II expression of antigen presenting cells (Moore et al. 2001). Deficiency of IL-10 in mice results in a form of inflammatory bowel disease that shows similarities to Crohn disease (Kuhn et al. 1993) and scar formation following normally scarless skin surgery illustrating the important role of IL-10 in modulating local inflammatory responses (Liechty et al. 2000).

The IL-10 families of cytokines consist of six related molecules that include IL-19, IL-20, IL-22, IL-24 and IL-26 in addition to the prototypic family member, IL-10. Like the related, type III IFNs, these cytokines also appear to play important roles at epithelial barriers to promote inflammation and tissue repair (Dudakov et al. 2015; Rutz et al. 2014). IL-19, IL-20 and IL-24 are produced primarily by myeloid lineage cells including macrophages and DCs within tissues. Their regulation is poorly understood, but TLR signaling appears to play an important role. While myeloid cells are capable of producing some IL-22, lymphoid cells represent the primary source of this cytokine. Major lymphoid producers of IL-22 include a subset of CD4+ T cells that have been termed “Th22” cells due to their abundant secretion of this cytokine along with γ/δ T cells and NK cells (Eyerich et al. 2009). In addition, the recently described innate lymphocyte-like cells (ILCs), especially the group 3 ILCs that reside within tissues and lack α/β or γ/δ TCR, appear to be a major source of IL-22. All of the IL-10 related cytokines have direct effects on the differentiation and/or function of epithelial cells. The effects of these cytokines,

however, show some differences across organs, and this specificity is likely due in part to variations in receptor expression. The receptor for IL-20 is found predominantly in skin, lung, ovarian and testicular epithelium. In contrast, the IL-22R is expressed across a range of epithelium of the kidneys and digestive system along with skin (Blumberg et al. 2001; Aggarwal et al. 2001). IL-22 plays an important in the homeostasis at epithelial surface affecting the population of commensal bacteria that inhabit these environments (Zenewicz et al. 2013). It also prevents systemic colonization by gut-resident bacteria (Sonnenberg et al. 2012). In addition, it appears that some pathogens such as *Salmonella enterica* subvar *typhimurium* can also subvert the role of IL-22 to change the commensal microbiome to its advantage (Behnsen et al. 2014). Like IL-10, IL-22 and related family members also appear to be important for regulating inflammation and repair of the barrier function at epithelial surfaces. Dysregulation of IL-22 is associated with pulmonary fibrosis (Simonian et al. 2010). IL-10 and related families have also been implicated in fibrosis within a variety of tissues including the lung, kidney, intestines and liver illustrating the role of these cytokines in maintaining the delicate balance between tissue injury and repair (Sziksz et al. 2015).

JAKs and STATs: The Core of Type I and Type II Cytokine Signaling

A relatively simple signaling pathway formed by the Janus family of tyrosine kinases (JAKs) and the signal transducers and activators of transcription (STAT) family of transcription factors is at the core of the mechanism by which type I and type II cytokines mediate their myriad of effects. The JAKs are comprised of 4 members termed JAK1, JAK2, JAK3 and TYK2. These kinases selectively bind to the cytoplasmic domains of different cytokine receptors through their N-terminus, and each cytokine receptor complex activates at least one or more JAKs. The specificity of cytokine signals is therefore determined in part by the various combinations of JAKs expressed and recruited by the individual cytokine receptor. JAK1, for example, is activated by IFNs as well as γ_c cytokines (i.e. IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). However, only IFN-γ from this group of cytokines activates JAK2 in addition to JAK1. In contrast, the IFN-α/βR selectively activates TKY2 along with JAK1. Cytokines that share gp130 (e.g. IL-6, IL-11, LIF, OSM, IL27) appear to all recruit JAK1, JAK2 and TYK2. For IL-6, JAK1 appears to be absolutely required, but the relative importance of the other JAKs to effective signaling is less clear.

In addition to the contribution of JAKs to signaling diversity, the seven STATs lead to further diversity in the cellular responses to different cytokines. STATs exist in cells in a latent form that is activated following binding to phosphorylated sites in cytoplasmic domains of cytokine receptors. Following binding, STATs are themselves phosphorylated by JAKs leading to their dissociation, dimerization and translocation to the nucleus where they bind to conserved sequences within DNA to activate transcription. Some STATs are capable of forming heterodimers increasing the complexity of signals that can be generated by this system. Table 3.1 shows examples of the JAKs and STATs activated by different cytokine receptors. While

the JAK-STAT signaling pathways is the primary signals generated by cytokine receptors, it is important to recognize that other pathways may also be activated by cytokine receptors such as PI-3-kinase through phosphorylation of the p85 subunit (Migone et al. 1998; Wills-Karp and Finkelman 2008).

Tumor Necrosis Factor (TNF) Family of Cytokines

The TNF family forms a structurally distinct group of cytokines and membrane-associated proteins that play central roles in the growth, survival and differentiation of cells (Ware 2013). Since TNF was initially described, over 19 TNF-related proteins have been identified to date as shown in Table 3.2. TNF and related family members are type II integral transmembrane proteins that share a conserved domain referred to as the TNF homology domain. This domain plays a critical role in TNF structure by allowing the molecule to assemble into trimers with 3-fold symmetry, which is essential to the functional form of the molecule (Locksley et al. 2001). Proteolytic cleavage of the membrane-proximal region of the molecule by TNF-α converting enzyme (TACE, also known as ADAM17), a membrane protease involved in cleaving many proteins, leads to the liberation of soluble TNF trimers that can then diffuse to surrounding cells to mediate the paracrine effects of these cytokines (Scheller et al. 2011).

Some TNF family members such as 4-1BB ligand (4-1BBL) bind to a single receptor; however, many TNF family members exhibit binding to multiple receptors creating significant complexity within this cytokine family. TNF has two distinct receptors that differ substantially in their signaling. TNFR1 contains a conserved death domain (DD) that connects this receptor to proteins within cells containing a death effector domain (DED). DED-containing proteins include the procaspase 8 and 10 molecules that link TNFR1 signaling to apoptotic cell death (Valmiki and Ramos 2009). In contrast, TNFR2 lacks a DD domain, but recruits TNF-receptor associated factors (TRAFs) that activate a variety of downstream pathways such as NF-kB that contribute to many of the effects of these cytokines (Ha et al. 2009). Beyond TNFR1 and TNFR2, 28 additional receptors belonging to the TNF receptor superfamily have been described as shown in Table 3.2. In addition to their ligands, many of the TNF receptor family members are also shed by TACE cleavage to yield soluble receptors. In addition to reducing the surface expression of the receptor, shedding of TNFR1 and TNFR2 may also be an important regulatory mechanism to control the bioavailability of TNF. This regulation has been used therapeutically through the generation of recombinant soluble TNFR2 fused to the Fc region of IgG1, which is marketed as Etanercept for the treatment of rheumatoid arthritis and other autoimmune diseases (Scott 2014).

The TNF family plays diverse roles throughout immunity and represents an area of intense investigation. TNF along with its most closely related cytokines, the lymphotoxins (LTs) and LIGHT appear to play critical roles in the communication between lymphoid cells and the tissues that they inhabit (Ware 2005). These cytokines, activated by many innate sensing systems already

Table 3.2 Tumor necrosis factor family and their receptors

TNF superfamily		TNF receptors	
Gene symbol	Common name	Gene symbol	Common name
TNFSF1A	TNF	TNFRSF1A	TNFR-1
TNFSF1B	Lymphotoxin-α	TNFRSF1B	TNFR-2
TNFSF3	Lymphotoxin-β	LTβR	LTβR
TNFSF4	OX40 Ligand (OX40-L, CD134)	TNFRSF4	OX40
TNFSF5	CD40 Ligand (CD40-L, CD154)	CD40	CD40
TNFSF6	Fas Ligand (Fas-L, CD95-L, CD178)	TNFRSF6	FAS, CD95
TNFSF7	CD27 Ligand (CD70)	TNFRSF6B	DcR3
TNFSF8	CD30 Ligand (CD30-L, CD153)	TNFRSF7	CD27
TNFSF9	4-1BB Ligand (4-1BB-L)	TNFRSF8	CD30
TNFSF10	TRAIL	TNFRSF9	4-1BB
TNFSF11	RANK Ligand (RANK-L, TRANCE)	TNFRSF10A	TRAILR-1, DR4
TNFSF12	TWEAK	TNFRSF10B	TRAIL-R2, DR5
TNFSF13	APRIL (TALL2)	TNFRSF10C	TRAIL-R3, DcR1
TNFSF13B	BAFF (BLYS, TALL1)	TNFRSF10D	TRAIL-R4, DcR2
TNFSF14	LIGHT	TNFRSF11A	RANK, TRANCE-R
TNFSF15	TL1A	TNFRSF11B	OPG, TR1
TNFSF18	GITR Ligand (GITR-L)	TNFRSF13B	TACI
EDA1	EDA1	TNFRSF13C	BAFF-R
EDA2	EDA2	TNFRSF14	HVEM, HveA, ATAR
		TNFRSF16	NGFR
		TNFRSF17	BCMA
		TNFRSF18	GITR
		TNFRSF19L	RELT
		TNFRSF19	TROY, TAJ
		TNFRSF21	DR6
		TNFRSF25	TRAMP, DR3, LARD
		EDAR1	EDAR
		EDA2R	EDA2R
		EGFLR1	EGFLR1, Tmem149

described, serve as important signals to promote inflammatory processes. Mice with a deficiency of LTα or the LT receptor show mostly profound defects in lymphoid tissues development with complete disruption or absence of normal lymphoid tissue architecture. While TNF or TNFR2 deficient mice show mostly intact lymphoid tissue, some lymphoid tissues such as the Peyer's patches within the intestinal mucosa are reduced or missing demonstrating the overlapping, but distinct functions of these cytokines. BAFF and APRIL, two closely related TNF family members that share receptors and are also capable of forming both homotrimers as well as heterotrimers of both molecules, play important roles in regulating B cell development and differentiation (Vincent et al.

2013). Fas ligand (FasL) and TRAIL along with TNF through its interaction with TNFR1 also play important effector and regulatory roles in the immune system by the selective induction of apoptotic cell death. The critical regulatory role of FasL and its receptor Fas in T cell biology is best illustrated by the severe autoimmunity and lymphoproliferation that occurs in mice (lpr and gld strains) and humans that are deficient in their expression. Numerous therapeutics that manipulate these molecules are already in the clinic such as etanercept and infliximab targeting TNF and many are currently under development such as agonist antibodies to 4-1BB, CD40, CD40L and BlyS (also known as BAFF) (Croft et al. 2013).

The IL-1 Family

The IL-1 family of cytokines includes seven proteins with pro-inflammatory effects that include IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ. These proteins all share structural similarities including the absence of a signal peptide, the presence of N-terminal pro-peptide sequence that is cleaved by serine proteases (e.g. caspase-1) and a conserved A-X-D sequence (A is any aliphatic amino acid; X is any amino acid; D is aspartic acid), which plays a role in the folding and tertiary structure of active IL-1 family members. The unique post-translational processing mechanisms involved in producing active forms of these cytokines and their MyD88-dependent receptor signaling were already discussed in the preceding section on the inflammasome.

The IL-1 family of cytokines bind to a group of related receptors for which there is varying nomenclature as shown in Table 3.3. Similar to other cytokine receptor families, many of the IL-1 receptor (IL-1R) family members are expressed as both transmembrane and soluble receptors with the latter likely playing important roles

Table 3.3 IL-1 family cytokines and their receptors

Gene symbol	Common name	IL-1 family nomenclature[a]	Receptor
IL1A	IL-1α	IL1F1	IL-1R1/IL-1RAP
IL1B	IL-1β	IL1F2	IL-1R1/IL-1RAP
IL1RN	IL-1Ra	IL1F3	IL-1R1
IL18	IL-18	IL1F4	IL-18R/IL-18RAP
IL36RN	IL-36Ra	IL1F5	IL-1RL2
IL36A	IL-36α	IL1F6	IL-1RL2/IL1RAP
IL37	IL-37	IL1F7	unknown
IL36B	IL-36β	IL1F8	IL-1RL2/IL1RAP
IL36G	IL-36γ	IL1F9	IL-1RL2/IL1RAP
IL1F10	*IL-38*[*]	IL1F10	unknown
IL33	IL-33	IL1F11	IL1RL1(ST2)/IL-1RAP

[a]Nomenclature suggested by Sims et al. (2001)
[*]This is a reserved name

in modulating and dampening the effects of these highly inflammatory cytokines. Several decoy receptors (IL-1R2 and IL-18BP) that lack cytoplasmic signaling domains as well as soluble receptor antagonists (e.g. IL-1R antagonist [IL-1RA] and IL-38) that block IL-1 binding and signaling through its receptor confer additional levels of regulation.

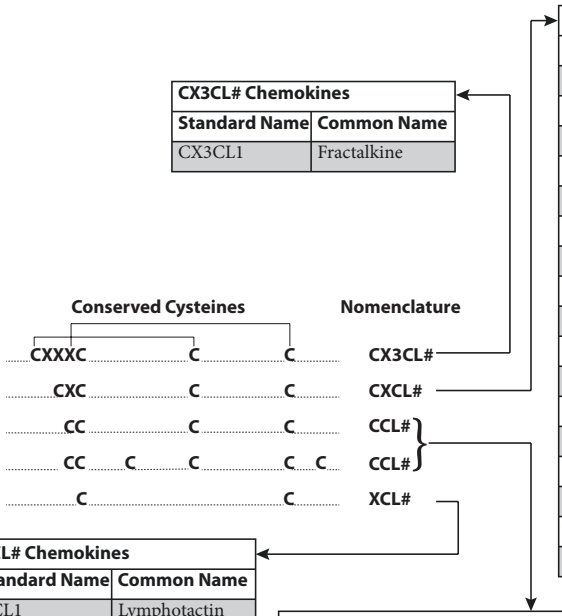
IL-1, the prototypic member of this cytokine family, is encoded by two genes that give rise to IL-1α and IL-1β. Both molecules bind to the same IL-1R and trigger similar biologic effects. Epithelial cells across a range of tissues that include the mucosa of the gastrointestinal tract, endothelium, kidney, lung and liver produce the IL-1α precursor protein. It is also produced by astrocytes within the central nervous system. In contrast, IL-1β is produced mainly by hematopoietic cells such as monocytes and tissue-resident macrophages. The key distinction between these two related cytokines is the mechanisms of activation and release. Upon necrotic cell death such as following tissue ischemia, IL-1α is released to initiate a rapid and robust inflammatory cascade that recruits myeloid cells such as neutrophils into the tissue in the absence of pathogens (so called sterile inflammation) (Chen et al. 2007; Rider et al. 2011). Thus, IL-1α serves as an "alarmin", like IL-33, to signal the presence of acute tissue injury initiating host defense and tissue repair. In contrast to IL-1β, which requires proteolytic cleave of the N-terminal sequence for biologic activity as already described, IL-1α is fully active in its precursor form. IL-1α also has a unique nuclear localization signal (NLS) that allows it to localize to the nucleus of the cell where it binds to DNA. This NLS also appears important for the differential release of active IL-1α following apoptotic compared with necrotic cell death. During apoptosis, the IL-1α remains bound to DNA where it remains unavailable for receptor binding and initiation of inflammation producing "silent" cell death. Following necrotic cell death, IL-1α rapidly translocates to the cytoplasm of the cell where it is released in active form to bind to the IL-1R to trigger inflammation (Cohen et al. 2010).

The IL-1 family of cytokines plays important biological roles in inflammation. Individuals who have genetic gain-of-function mutations within the NLRP3 (also known as cryopyrin) inflammasome, which processes IL-1β precursor into mature protein, exhibit a constellation of inflammatory syndromes known as cryopyrin-associated periodic syndromes (CAPS) associated with elevated production of IL-1β. CAPS range from more mild syndromes like rash, conjunctivitis, arthralgia with fever to more severe syndromes such as neonatal onset multisystem inflammatory disease that is associated with uncontrolled autoinflammation across multiple tissues resulting in rashes, severe arthritis and meningitis that leads to neurologic damage (Shinkai et al. 2008). The response of CAPS to IL-1 or IL-1R blockade by drugs such as canakinumab (anti-IL-1β), rilonacept (an IL-1 binding protein) or anakinra (IL-1RA) demonstrate the central role that IL-1β plays in these disorders (Kone-Paut and Piram 2012). IL-1 has also been implicated in diseases including atherosclerosis and obesity-related metabolic syndrome supporting inflammatory etiologies to these diseases (Stienstra et al. 2012; Freigang et al. 2013).

Chemokines

Chemokines (chemotactic cytokines) represent a large and diverse class of small cytokines that play central roles in the control of cellular migration and the spatial distribution of cells within tissues. The chemokine system is a complex intercellular signaling system that includes approximately 50 individual chemokines that share a tertiary structure determined by conserved cysteine residues within their amino acid sequence. The majority of chemokines contain at least four, conserved cysteine residues with some containing six and only the chemokines, lymphotactin α and β containing two conserved cysteine residues. The location of these conserved cysteine residues within the amino acid sequence forms the basis for the current nomenclature used for this large class of molecules as shown in Fig. 3.3. The enormous diversity of chemokines is complemented by over 20 chemokine receptors that belong to the rhodopsin-like seven-transmembrane receptor family of G-protein coupled receptors (GPCRs). The majority of these receptors deliver signals to cells G-protein signaling; however, a group of atypical receptors also exist that appear to modulate chemokine gradients through ligand binding in the absence of signaling such as the Duffy antigen receptor for chemokines (DARC) (Dawson et al. 2000; Pruenster et al. 2009). The genetic diversity of chemokines within species is further complicated by their variation across species. While zebrafish have over 63 different chemokines compared with 48 known chemokines in humans, only two CXC chemokines in humans, CXCL12 and CXCL14 have orthologs in fish. In fact, none of the other CXC chemokines have known orthologs in vertebrates outside of mammals suggesting that chemokine evolution has occurred in lock step with the evolution of the vertebrate immune system (Nomiyama et al. 2010; DeVries et al. 2006). Diversity is even observed within humans. The genetic locus containing the macrophage inflammatory protein (MIP)-like chemokines, CCL3-like (CCL3L) and CCL4-like (CCL4L) shows significant copy number variation across individuals that has been associated with outcomes in infection with human immunodeficiency virus (HIV) (Colobran et al. 2010).

An in-depth discussion of the chemokine family and their functional roles in immunity are well beyond the scope of this chapter. Readers are referred to comprehensive reviews devoted to the subject (Griffith et al. 2014). In general, chemokines can be functionally grouped into two main functional categories based upon whether they are largely involved in recruiting and localizing cells within tissues during inflammatory states where they help promulgate the inflammatory processes or during non-inflammatory conditions (i.e. homeostasis). CCR7, the chemokine receptor for CCL19 and CCL21 is normally expressed by naïve and memory T cell subsets. The constitutive expression of CCL21 by high endothelial venules (HEVs) within the T cell rich zones of the lymph node and CCL19, produced by fibroblastic reticular cells (FRCs) within the lymph node and delivered to the luminal surface of the endothelium by transcytosis help to recruit CCR7-expressing T cells into the paracortical regions. Driven by gradients of these chemokines, T cells subsequently scan for cognate antigen derived from the draining lymph by antigen presenting cells. The continued exposure of CCR7 to its ligands induces down-regulation of this



CX3CL# Chemokines

Standard Name	Common Name
CX3CL1	Fractalkine

CXCL# Chemokines

Standard Name	Common Name
CXCL1	GROα; MGSA
CXCL2	GROβ; MIP-2
CXCL3	GROγ; MIP-2β
CXCL4	Platelet factor 4
CXCL5	ENA-78
CXCL6	GCP-2
CXCL7	NAP-2
CXCL8	IL-8
CXCL9	Mig
CXCL10	γIP-10
CXCL11	I-TAC
CXCL12	SDF-1α
CXCL13	BLC
CXCL14	BRAK
CXCL15	Lungkine
CXCL16	SR-PSOX
CXCL17	

XCL# Chemokines

Standard Name	Common Name
XCL1	Lymphotactin
XCL2	

CCL# Chemokines

Standard Name	Common Name	Standard Name	Common Name
CCL1	I-309	CCL15	HCC-2
CCL2	MCP-1;	CCL16	HCC-4
CCL3	MIP-1α	CCL17	TARC
CCL4	MIP-1β	CCL18	PARC
CCL5	RANTES	CCL19	ELC
CCL6	C10; MRP-1	CCL20	MIP-3α; LARC
CCL7	MCP-3	CCL21	SLC
CCL8	MCP-2	CCL22	MDC
CCL9	MRP-2; MIP-1γ	CCL23	MPIF-1
CCL10	(reserved)	CCL24	Eotaxin-2
CCL11	Eotaxin	CCL25	TECK
CCL12	MCP-5	CCL26	Eotaxin-3
CCL13	MCP-4	CCL27	CTACK
CCL14	HCC-1	CCL28	MEC

Fig. 3.3 Nomenclature of chemokines is based on the presence and location of conserved cysteine residues (C) in the molecules, with X representing intervening amino acids. Many of the chemokines have common names that appear abundantly in the literature

chemokine receptor eventually allowing T cells to migrate towards other chemokine gradients such as that produced by S1P1 that lead the T cells into the efferent lymph for draining back into the general circulation for recruitment into other lymph nodes (Masopust and Schenkel 2013). Disruption of these chemokine signals by genetic loss leads to substantial alterations in immunity. Mice with CCR7 deletion show disruptions in normal lymph node morphology with markedly reduced numbers of

T cells within the paracortical regions. These morphologic changes are associated with delayed antibody responses as well as defects in T cell mediated contact hypersensitivity demonstrating the critical importance of normal T cell trafficking controlled by chemokines such as CCR7 in immunity (Forster et al. 1999). In contrast to normal homeostasis, many chemokines are critical for propagating inflammation as well promoting its resolution by orchestrating the recruitment of immune cells into tissues. Tissue resident macrophages, endothelial cells and other cells within tissues, following TLR signaling and NF-κB activation, produce IL-8 (CXCL8). This potent pro-inflammatory cytokine helps to recruit neutrophils, monocytes and other important inflammatory cells into tissues to promote inflammatory states across tissues (Yoshimura 2015).

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Chapter 4
Development of Immune System Organs

George A. Parker

Abstract In addition to the direct, indirect, nutrition- and stress-related histologic alterations encountered in immune system organs of adult animals in toxicology studies, in juvenile toxicology studies the pathologist and toxicologist must also be concerned with the influence of normal postnatal development on the histologic appearance and functional attributes of immune system tissues. In addition to direct age-related differences in histomorphology, the postnatal development of the immune system is partially controlled by environmental factors, thus test article-related influences on non-immune organs or functions, e.g., the microbiome of the gastrointestinal tract, may have secondary effects on immune system organs. It is important that pathologists and toxicologists have a thorough understanding of the normal embryological and postnatal development of immune system organs in order to prevent misinterpretation of these normal changes as xenobiotic-associated. This chapter is focused on histomorphological rather than physiological alterations, though the intent certainly is not to dismiss the importance of traditional immunological assays in the detection of xenobiotic-associated influences. The laboratory rat is commonly used in nonclinical toxicology studies, thus the rat is used as the major example of the processes that occur during the postnatal development of immune system organs. An attempt is made to provide cursory information relating developmental and homeostatic changes in the rat as compared to those seen in other commonly used lab animals and humans, but a thorough presentation of these changes in humans and all animal species is beyond the practical scope of this Chapter.

Keywords Embryology • Postnatal development • Embryogenesis • Organogenesis • Histology • Rats • Immune system

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Abbreviations

AGM	Aorta-gonad-mesonephros region
APC	Antigen-presenting cell
ARR	Antigen-retaining reticulum
BALT	Bronchus-associated lymphoid tissue
B-1 cells	B lymphocytes that also have a T cell marker
BLA	Basic lead acetate (fixative)
BTLA	B and T lymphocyte attenuator
CD	Clusters of differentiation
CD-L	Clusters of differentiation ligand
CD3	Immunohistochemistry marker for T cells
CD45RA	Immunohistochemistry marker for B cells
CD80/86	B7-1 and B7-2 costimulatory molecules, respectively
CINC	Cytokine-induced neutrophil chemoattractant
CLP	Common lymphocyte progenitor
CP	Cryptopatches of small intestine
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DAB	Diaminobenzidine
DART	Developmental and reproductive toxicology
DC	Dendritic cell
DHEAS	Dehydroepiandrosterone sulfate
DHEA	Dehydroxyepiandosterone
DIT	Developmental immunotoxicology
DTH	Delayed-type hypersensitivity
EMH	Extramedullary hematopoiesis
FAE	Follicle-associated epithelium
FC	Fragment, crystallizable
FCR	Fragment, crystallizable receptor
FDC	Follicular dendritic cells
GALT	Gut-associated lymphoid tissue
GC	Germinal center
G-CSF	Granulocyte colony stimulating factor
GD	Gestation day
H&E	Hematoxylin and eosin (histologic stain)
HEV	High-endothelial venules
HP-DC	Hematopoietic dendritic cell
HSC	Hematopoietic stem cell
IACUC	Institutional Animal Care and Use Committee
iBALT	Inducible bronchus-associated lymphoid tissue
ICOS	Inducible T cell co-stimulator
IEL	Intraepithelial lymphocyte population of small intestine
Ig	Immunoglobulin
IHC	Immunohistochemical
IL	Interleukin

ILF	Individual lymphoid follicle of small intestine
KLH	Keyhole limpet hemocyanin
Ki67	Proliferation marker for immunohistochemistry
LPF	Lipopolysaccharide
LTβ	Lymphotoxin β
LTβR	Lymphotoxin β receptor
LTi	Lymphoid tissue inducer (cells involved in lymphoid tissue embryogenesis)
LTo	Lymphoid tissue organizer (cells involved in lymphoid tissue embryogenesis)
M cells	Microfold cells
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MALT	Mucosa-associated lymphoid tissue
MC	Mast cells
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1α (chemokine)
MODC	Monocyte-origin dendritic cell
MZ	Marginal zone
MZB	Marginal zone B cells
NALT	Nasopharynx-associated lymphoid tissue
NETs	Neutrophil extracellular traps
NK	Natural killer cell
NKT	NK cell with T cell marker(s)
OX-2	Immunohistochemistry marker for follicular dendritic cells
OX-4	Orexin type 1
OX-4L	Orexin type 1 receptor
PALS	Periarteriolar lymphoid sheath
PD	Programmed cell death
pDC	Plasmacytoid dendritic cell
PD-L	Programmed cell death ligand
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphoinositide 3 kinase
PNad	Peripheral node addressin (cell adhesion molecular)
PND	Postnatal day
PP	Peyer's patches
RANTES	Regulated on activation, normal T cell expressed and secreted
TCR	T cell receptor
TFH	T follicular helper cells
TFR	T follicular regulatory cells
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNP-KLH	Trinitrophenyl conjugated to keyhole limpet hemocyanin
TNP-LPS	Trinitrophenyl conjugated to lipopolysaccharide
TNP-Ficoll	Trinitrophenyl conjugated to Ficoll
TREM	Recruitment of triggering receptor expressed on myeloid cells
VCAM	Vascular cell adhesion molecule

4.1 Introduction

Microscopic examination of histologic sections of multiple organs and tissues, including organs of the immune system, is a basic component of the nonclinical safety assessment of candidate drugs as well as agricultural and industrial chemicals. Traditional nonclinical toxicology studies were performed in young adult animals of multiple species, with the laboratory rat being the primary 'toxicology animal'. Evolving recognition of the dissimilarities between adults and children has brought the field of juvenile toxicology to the forefront of the overall nonclinical toxicity assessment. The histomorphology of adult laboratory animals is well-known to toxicologic pathologists through years/decades of experience, but there has been less opportunity for pathologists to become familiar with the details of histomorphology in juvenile animals. The primary goal of this Chapter is to provide details of the postnatal development of immune system organs in laboratory rats. Secondary goals include review of antenatal organogenesis in rats, and comparisons of postnatal development in rats, mice and humans.

4.2 Embryogenesis and Organogenesis of the Immune System Organs

The morphological and functional evolution of immune system organs in mammals is a continual process that commences early in embryonic development, reaches maximum development at some point in early adulthood, maintains at a plateau for an indeterminate period, and then declines. At birth the mammal transitions from the protected, fully supported status of the fetus to the independent status of the newborn animal, which results in altered function and structure of multiple organs. In the case of immune system organs, birth exposes the immune system to a plethora of antigens that stimulate secondary immune system organs to develop and maintain the structural and functional characteristics that we view as 'fully developed'. At the time of birth the immune system organs are at different stages of development, thus there is variation in the morphologic appearance of the organs. In order to fully appreciate the significance of the steps in postnatal development and maturation of immune system organs, it is helpful to have a general understanding of the embryonic events that lead to immune organ status at the time of birth. The examples used in this Chapter are largely drawn from the laboratory rat, which is commonly used in toxicology studies.

Cellular elements of the specific immune system arise from self-regenerating hematopoietic stem cells that have the ability to differentiate into all blood cells, including erythrocytes, lymphocytes/NK cells, mast cells, megakaryocytes, and the various subpopulations of granulocytes. The first manifestation of the hematopoietic system consists of large primitive red blood cells that are produced in blood islands of the yolk sac soon after implantation of the embryo (Palis and Yoder 2001). The first

HSCs are derived from the wall of the yolk sac (Moore and Metcalf 1970). Cells from fetal mouse yolk sacs at GD 7–8 give rise to functional macrophages in vitro (Cline and Moore 1972) (8), and mouse yolk sac cells at GD 8 are able to generate pure erythrocytic, megakaryocytic and granulocytic colonies as well as mixed colonies (Moore and Metcalf 1970). There has been a long-standing hypothesis that HSCs originate in the yolk sac, migrate to the liver, and eventually seed the bone marrow (Moore and Metcalf 1970). This classical sequence of events has been challenged, based on evidence that HSCs are present in the aorta-gonad-mesonephros (AGM) region of the developing embryo before their appearance in the yolk sac and liver (Sonoda et al. 1983; Harrison et al. 1979; Muller et al. 1994; de Bruijn et al. 2000).

Regardless of the exact origin of the HSCs, the earliest committed hematopoietic progenitors in the embryo are derived from uncommitted HSCs in the AGM (Cumano et al. 1996; Liu and Auerbach 1991; Ohmura et al. 1999). This is followed by vascular dissemination of primitive hematopoietic precursor cells to the liver and spleen, where they begin differentiation into hematopoietic lineages, and then seed the developing primary (thymus and bone marrow) and secondary (spleen and lymph nodes) lymphoid organs (Burns-Naas et al. 2008). Primitive lymphoid precursor cells arise from a split in the HSC population into a lymphocyte/NK cell line and a myeloid cell line that produces the remaining blood cells (neutrophils, eosinophils, basophils, monocytes/macrophages, erythrocytes and megakaryocytes). T cell progenitors emerge earlier than B cell progenitors in the liver of mice (Kawamoto et al. 2000).

Recent experimental evidence suggests there is a difference in the developmental sequence of blood cells in the fetus versus the adult. In the fetus, hematopoietic stem cells progress to multipotent cells, which further differentiate into oligopotent and, finally, unipotent cells, but in adults there is evidence that HSCs differentiate into a multipotent cell type that directly gives rise to megakaryocytes, and then progresses to the unipotent final erythroid, myeloid, and lymphoid/monocytoid cell types without passing through the oliogopotent intermediary (Notta et al. 2016).

4.2.1 Bone Marrow and Liver

From mid-gestation onwards the liver is the primary site of hematopoiesis in the mouse, and available evidence suggests the same progression in the rat. Mineralization of long bones with formation of marrow cavities near the end of gestation is the final stage in the embryonic development of hematopoiesis. Migration of hematopoietic cells into the marrow cavity starts at GD17.5 in mice (Kincade 1981). Our studies in rats (unpublished) have shown the liver to be a major site of hematopoiesis at GD15 (Fig. 4.1), followed at GD 20 by a reduction of hepatic hematopoiesis (Fig. 4.2) and early hematopoiesis in the marrow cavity of bones (Fig. 4.3).

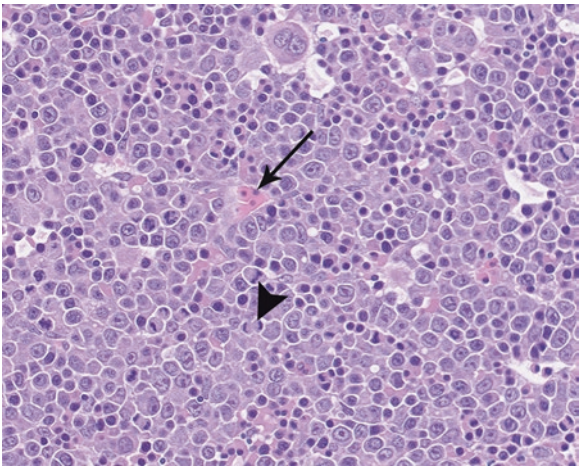

Fig. 4.1 Liver of rat at GD 15 consists of a sheet of hematopoietic cells intermixed with primitive hepatocytes. At this stage of development the liver lacks the hepatocellular cords and plates that characterize liver tissue in adults. Note the nucleated fetal erythrocytes (*arrow*) and numerous mitotic figures (*arrowhead*). Hematoxylin and eosin stain, 40× objective magnification

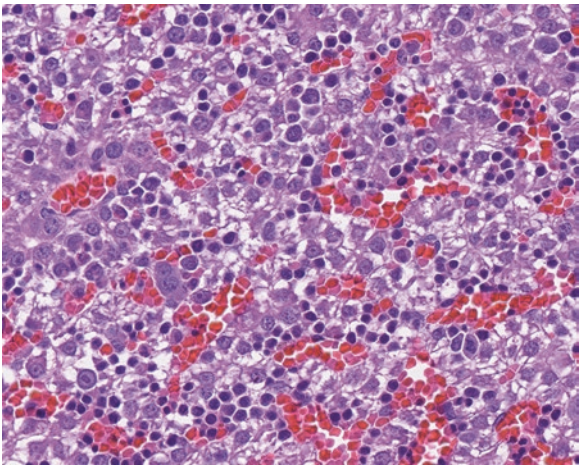

Fig. 4.2 Liver of rat at GD 20. Hepatocytes have highly vacuolated cytoplasm due to glycogen content, and form indistinct cellular cords. Hematopoietic cells commonly exist in irregular linear arrays that correspond to developing hepatic sinusoids. Hematoxylin and eosin stain, 40× objective magnification

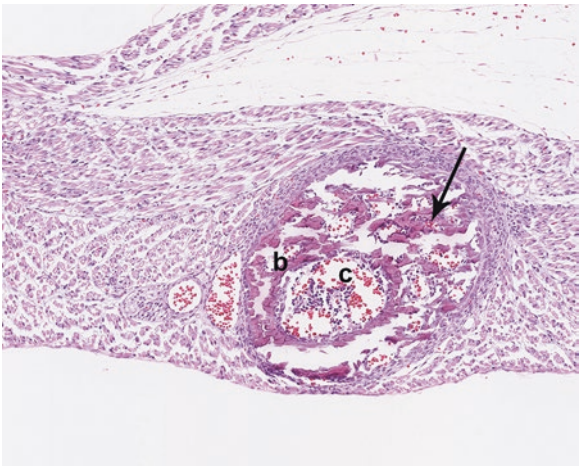

Fig. 4.3 Rib of rat at GD 20. A modest population of hematopoietic cells is present in the central marrow cavity (*c*) being formed as the cortical bone (*b*) is mineralized. Mineral spicules (*arrow*) are retained in this non-decalcified specimen. Hematoxylin and eosin stain, 10× objective magnification

4.2.2 Thymus

The thymus is the primary site of T cell selection and maturation from the early juvenile state until the thymus involutes sometime between puberty and mid-adulthood. The thymus of most species develops from pharyngeal pouches III and IV, and migrates caudally along with the thyroid and parathyroid glands (Kuper et al. 2013) to lie in the anterior mediastinal space. There is some disagreement regarding the terminology of the pouches in the rat, with some investigators maintaining that the rat does not have a true pharyngeal pouch IV (Harland 1940).

The thymus is distinct from other lymphoid organs in that the stroma develops primarily from epithelial cells (Picker and Siegelman 1999), as compared to the mesenchymal origin of the stroma of other lymphoid organs. The rudimentary thymic epithelial component of humans arises from (a) endoderm of the third pharyngeal pouch, (b) ectoderm of the corresponding branchial clefts, and (c) stromal cells of the associated mesenchyme from the pharyngeal arch, the latter being derived from the neural crest. Interaction between epithelia of endodermal and ectodermal origin appears necessary for the development of the intact thymus. Neural crest involvement is necessary for development of the thymus (Bockman and Kirby 1985), particularly the thymic medulla (Brelinska et al. 2001). Defects in the neural crest contribution are involved in human clinical conditions such as DiGeorge syndrome (Hong 1991). In the 'athymic' nude mouse, the ectoderm of the third branchial cleft involutes after GD11.5, further development of the endoderm is halted, and the thymus does not progress to form an intact lymphoid organ (Cordier and Haumont 1980).

The epithelial-based thymic stroma and lymphoid cell population have separate origins that occur early in embryonic development. The primordial epithelial components of the thymus receive HSC at approximately GD11 in the mouse (Shortman et al. 1998), before the thymus is vascularized. Thereafter a codependent development of epithelial and lymphoid elements results in the final structure. The incoming prethymic lymphoid cells are first derived from the earliest hematopoietic centers in the yolk sac and/or AGM (Moore and Owen 1967; Owen and Ritter 1969), then the fetal liver (Ema and Nakauchi 2000), and postnatally from the bone marrow (Le Douarin et al. 1984). In mouse models, influx of lymphoid cells into the thymus occurs as cyclic waves of progenitor cells at programmed times of embryonic development, leading to self-perpetuating and differentiating populations of thymocytes (Fontaine-Perus et al. 1981). This pattern of cyclic introduction of precursor cells followed by group maturation of cells extends into postnatal life (Morin et al. 1992). Successful maturation and exit of one population of mature naïve T cells affords space and support for a subsequent population of entry-level T cells to enter the thymus near the corticomedullary junction. The various populations of maturing thymocytes are not distinguishable in routinely stained histologic sections, though a larger population of mitotically active cells is sometimes discernible in the subcapsular zone. However, the clustering of related thymocyte iterations may be visible in immunohistochemical stains for apoptosis. The double-positive (DP) stage of thymocyte generation is exquisitely sensitive to the glucocorticoids that are released as part of the stress response

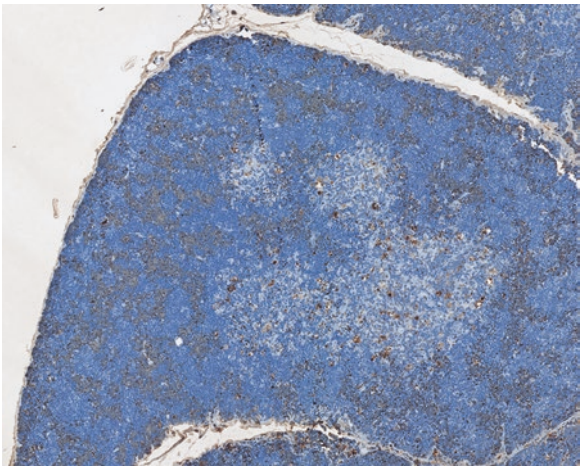

Fig. 4.4 Thymus of a rat following administration of dexamethasone. Note the zones of *brown-stained apoptotic cells* intermeshed with zones of *unstained cells*, indicating contiguous groups of cells with group-related differences in sensitivity to glucocorticoid-induced apoptosis. Developing thymocytes at the double-positive (DP) stage are known to be exquisitely sensitive to glucocorticoids. Terminal UDP-labeled end-link (TUNEL) staining with diaminobenzidine chromagen and hematoxylin counterstain, 10× objective magnification

(Boldizsar et al. 2006). Experimental administration of glucocorticoids may reveal distinct patches of apoptotic thymocytes with intervening areas of non-apoptotic cells, thus indirectly indicating the location of cells in the DP stage (Fig. 4.4).

In comparison to the mouse and human, less attention has been given to the embryological development of the thymus of the rat. However, it is known to involve similar concurrent changes in the mesenchymal and epithelial components (Harland 1940). Mesenchymal cell activity is divided into two overlapping periods: early lymphocyte immigration (GD14–16.5) and lymphocyte multiplication and emigration (post-GD16). After GD16.5 the rate of lymphoid cell immigration into the thymus of the rat declines and the number of lymphocytes in the thymus is increased primarily by mitotic division.

Simultaneous with the mesenchymal (lymphocytic) development, the ventral diverticulum of pouch III develops into the epithelial stroma of the thymus. During the later stages of embryonic thymic development in the rat there is a conspicuous subcapsular marginal zone where the mature lymphocyte population is not sufficiently intense to obscure the background reticulum (Harland 1940) (Fig. 4.5). This peripheral marginal zone was sometimes faintly discernible in the postnatal thymus (Harland 1940), an observation that was confirmed in our studies. Distinct medullary zones were present in the thymus of rats at GD16.5 (Harland 1940). Immunohistochemical studies indicate the thymic medulla has a prominent population of cells that express neuron-specific enolase, consistent with the neural crest contribution to the thymic medulla (Brelinska et al. 2001).

While the thymic stroma of most species is thought to be derived from a combination of endoderm of the third pharyngeal pouch, ectoderm of the corresponding branchial clefts, and mesenchyme of the pharyngeal arch, observations in the rat

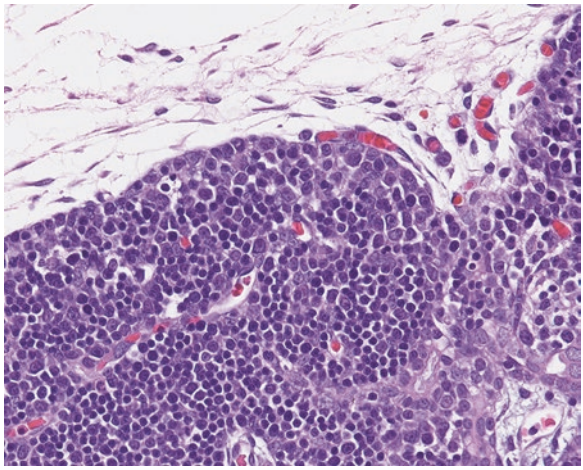

Fig. 4.5 The superficial aspect of the thymus of a rat at GD 20 has a prominent population of large, irregularly *round cells* that represent the superficial proliferative zone. A zone of smaller, more mature lymphocytes is present in the deeper aspect of the thymic cortex at the *bottom* of the image. Hematoxylin and eosin stain, 40× objective magnification

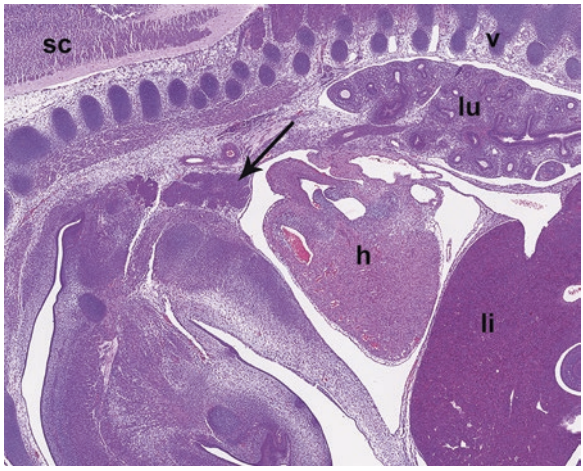

Fig. 4.6 Thoracic cavity of rat at GD 15 has thymus (*arrow*) in close approximation to the anterior extent of the thoracic cavity. *H* = heart, *li* = liver, *lu* = lung, *sc* = spinal cord, *v* = vertebra. Hematoxylin and eosin stain, 2.5× objective magnification

suggest there is no contribution from the ectoderm of the branchial clefts (Harland 1940). In the embryonic rat the ectodermal cervical vesicle was identified as a constant structure until GD16, after which it was present in a degenerative condition that would be unlikely to contribute to further development (Harland 1940).

The thymus reaches its final anatomic destination at approximately GD15 in the mouse. The exact sequence of cellular migration and maturation is unknown in the rat, but is presumed to be similar to that seen in the mouse. Our observations in rats indicate the thymus is in close proximity to the final destination by GD15 (Fig. 4.6), consisting of a uniform population of cells with no internal histologic structure (Fig. 4.7). By GD 20 the thymus has reached its final destination in the anterior mediastinum (Fig. 4.8), and an early manifestation of corticomedullary distinction is evident (Fig. 4.9). At GD 20 the thymic lymphoid cell population exhibits strongly positive immunohistochemical staining for CD3, a T cell marker (Fig. 4.10), and many thymic cortical cells exhibit positive immunohistochemical staining for Ki67, a proliferation marker (Fig. 4.11).

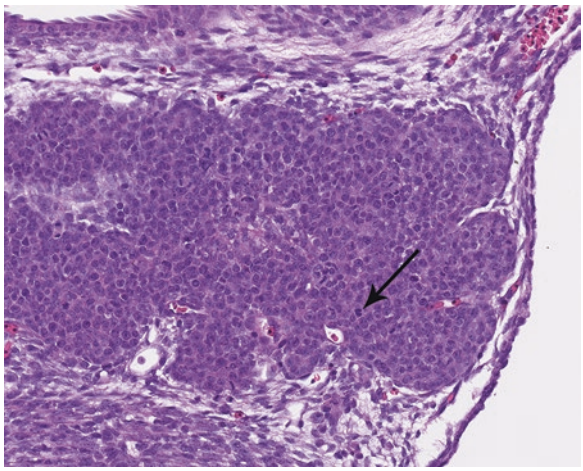

Fig. 4.7 Higher magnification of thymus shown in Fig. 4.6. Note absence of corticomedullary differentiation and numerous mitotic figures (*arrow*). Hematoxylin and eosin stain, 20× objective magnification

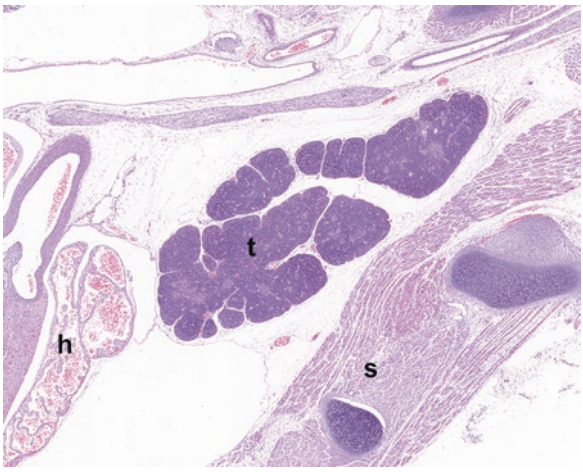

Fig. 4.8 Thymus (*t*) of a rat at GD 20 is in its final location in the anterior mediastinal space, immediately anterior to the heart (*h*) and dorsal to the sternum (*s*). Hematoxylin and eosin stain, 2.53× objective magnification

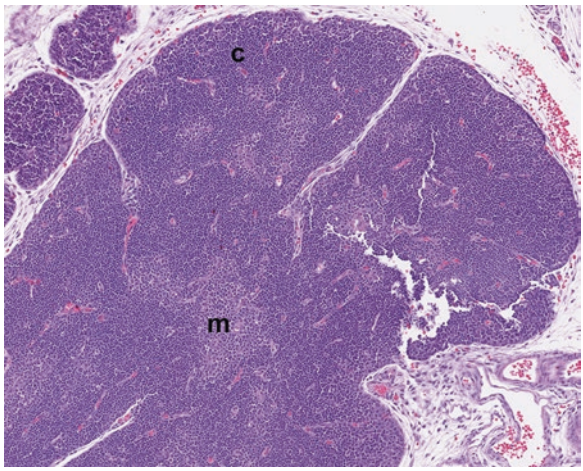

Fig. 4.9 The thymus of a rat at GD 20 has discernible cortical (*c*) and medullary (*m*) regions, though the demarcation between cortex and medulla is not as sharply defined as in the thymus of adult rats. Hematoxylin and eosin stain, 10× objective magnification

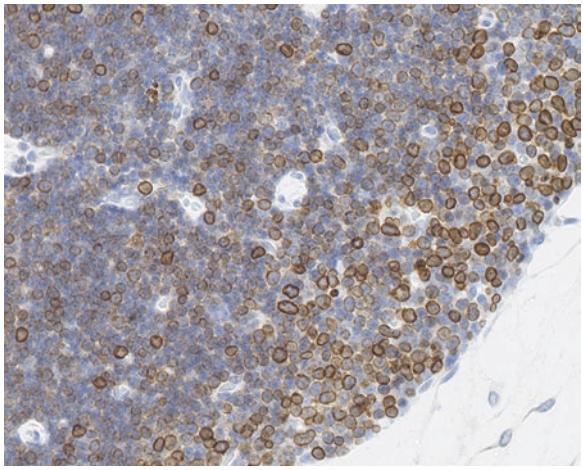

Fig. 4.10 Cortical region of the thymus of a rat at GD 20 shows strongly positive immunohistochemical staining (*brown*) for CD3. Note the concentration of positively stained cells in the superficial aspect of the thymus. CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 40× objective magnification

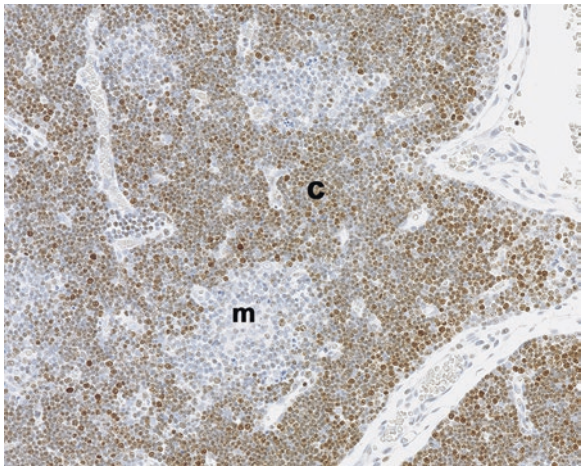

Fig. 4.11 The thymus of a rat at GD 20 shows intense proliferative activity in the cortex (*c*), as evidenced by *brown staining* of the majority of cells in the cortex. Note the sparse proliferative activity in the medulla (*m*). Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

Observations based on histologic features of the thymus are supported by cytologic features. Cytologic analysis of the thymus of rats revealed lymphoid cell proliferation started at GD14, remained high throughout embryogenesis to reach a maximum level at GD18, decreased soon after birth, and increased again at PND 21 (Aboussaouira et al. 1988). A decrease in thymic lymphoid cell proliferation antedated the onset of sexual maturation, and was marked in aged rats (Aboussaouira et al. 1988).

4.2.3 Spleen

The spleen is much more complex than the thymus from the perspective of the histopathologist. The spleen has both non-immunological and immunological functions, the latter largely concentrated in 'white pulp' that consists of the periarteriolar lymphoid sheath (PALS), lymphoid follicles, and marginal zone. Though the PALS is considered

to be the 'T cell zone' of the spleen, in reality the PALS has an inner zone that is populated primarily by T cells and an outer zone that is populated primarily by B cells, with a minor population of T cells and macrophages. Follicles typically develop at the junction of the T cell and B cell regions, though the exact site of origin may be difficult to determine histologically in large, fully developed follicles. Surrounding the PALS and follicles is the marginal zone (MZ), which is populated by marginal zone macrophages, marginal zone metallophilic macrophages, and a B cell population that includes unique cells known as marginal zone B cells (MZBs). Marginal zone B cells have special phenotypic markers (surface $IgM^{+}IgD^{-}$) and specialized functions that include a high level of reactivity to polysaccharide antigens (Hardy 2013).

Embryological development of the spleen commences early in gestation but, unlike the thymus, full development of the immune system components of the spleen is dependent on environmental stimulation. In the human fetus, the stomach is attached to the dorsal body wall by the dorsal mesogastrium and to the ventral body wall by the ventral mesogastrium. The spleen primordium appears in the fifth week of gestation as a mesodermal proliferation between the two leaves of the dorsal mesogastrium (Sadler 1995). The dorsal mesogastrium later fuses with the peritoneal wall, resulting in splenic attachment to the dorsal body wall in the region of the left kidney by the dorsal lienorenal ligament and to the stomach by the gastrolienal (or gastrosplenic) ligament.

The spleen of the mouse forms from the fusion of (typically) five aggregates of mesodermal tissue in the dorsal mesogastrium (Kaufman and Bard 1999). As a result of this segregated embryological origin, the splenic artery typically forms five major branches within the spleen. On occasion one of the mesodermal aggregates fails to associate with the remaining aggregates, resulting in an isolated nodule of splenic tissue that has a separate vascular supply. These splenic nodules are called 'ectopic' or 'heterotopic' spleen, which is something of a misnomer. The isolated splenic nodules are not in an abnormal location, as suggested by the terminology, but instead represent a fusion defect.

In mice, HSC and lineage-restricted hematopoietic progenitor cells are found in the fetal spleen at approximately GD13 (Landreth 1993). Splenic hematopoietic cells persist into postnatal and adult life, where they support development of myeloid and erythroid elements, but not lymphoid elements (Paige et al. 1981). This suggests the bone marrow hematopoietic environment is necessary for lymphocytopoiesis in mice (Graziano et al. 1998; Parrott et al. 1966). As with other secondary lymphoid organs, development of the spleen is somewhat dependent on antecedent development of the thymus and bone marrow, as these primary lymphoid organs provide the cellular seed-stock from which splenic immune cell populations develop.

Spleen embryogenesis in the mouse is similar to that in humans, and our observations suggest a similar developmental pattern in the rat. At GD15 the primordial spleen of the rat is contained within the dorsal mesogastrium (Fig. 4.12), and has no discernible internal substructures (Fig. 4.13). At GD20 the spleen of the rat lies adjacent to the stomach, and has the triangular cross-sectional profile that is seen in adult rats (Fig. 4.14). At GD 20 the spleen of the rat has arterioles (Fig. 4.15) that will later be surrounded by the lymphoid elements of PALS, but at this stage there is no histologic evidence of PALS or other lymphoid elements. At GD 20 the rat spleen has a moderate diffuse population of $CD45RA^{+}$ cells, consistent with B cells or B cell precursors from

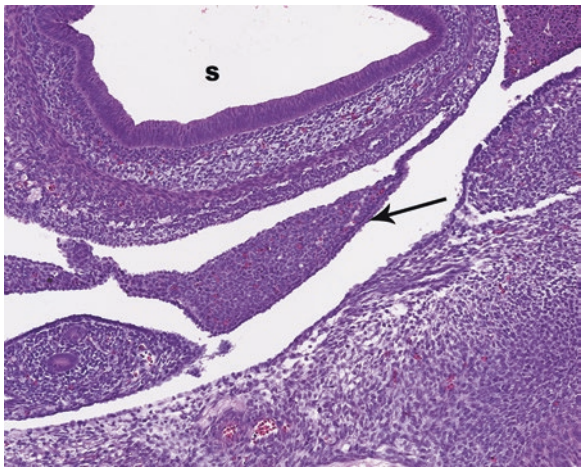

Fig. 4.12 The spleen (*arrow*) of a rat at GD 15 is in close approximation to the stomach (*s*). Note the gastrolienal (gastrosplenic) and lienorenal ligaments that anchor the ends of the developing spleen to the surface of the stomach and perirenal soft tissue, respectively. Hematoxylin and eosin stain, 10× objective magnification

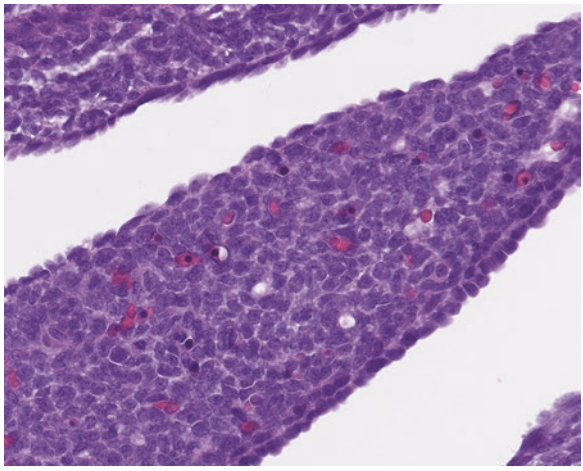

Fig. 4.13 Higher magnification of the spleen shown in Fig. 4.11. Note the absence of internal structure. Hematoxylin and eosin stain, 40× objective magnification

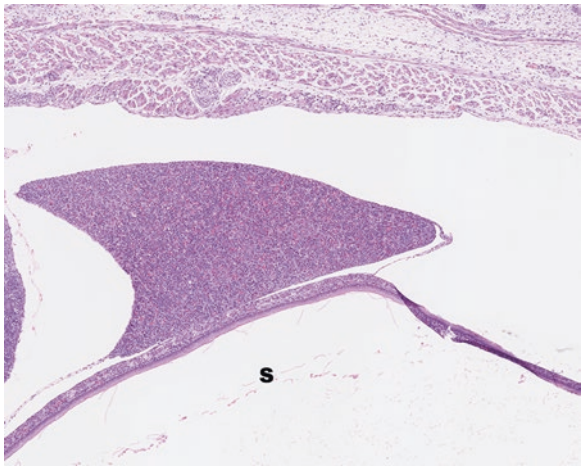

Fig. 4.14 The spleen of a rat at GD 20 has the triangular cross-sectional profile that is typical of the spleen of adult rats, but lacks histologic evidence of internal structure. *S* = stomach. Hematoxylin and eosin stain, 5× objective magnification

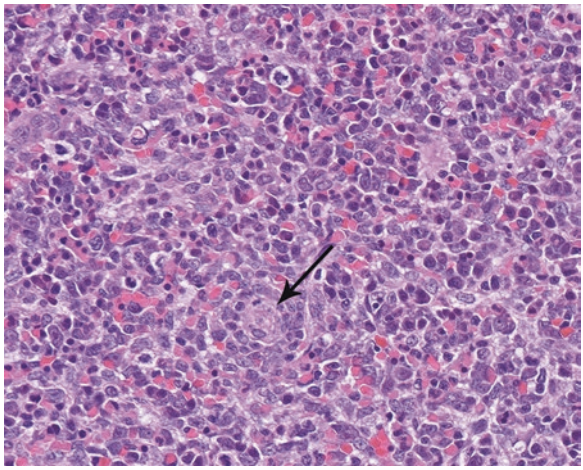

Fig. 4.15 Higher magnification of the spleen shown in Fig. 4.13. Note the arteriole (*arrow*) surrounded by a sheet of intermixed cell types, with no evidence of the periarteriolar lymphoid sheath that characterizes the spleen of adult rats. Hematoxylin and eosin stain, 40× objective magnification

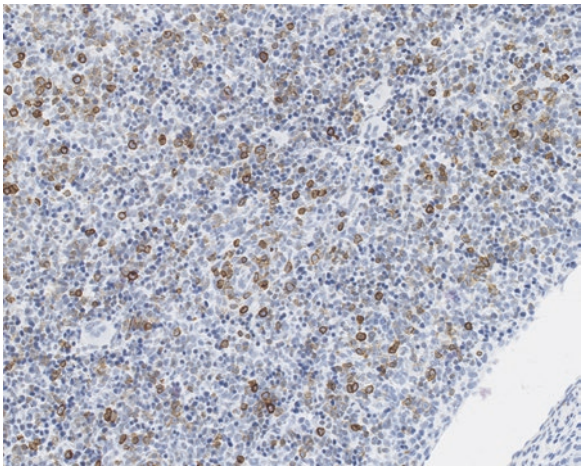

Fig. 4.16 Immunohistochemical staining for a B cell marker performed on the spleen of a rat at GD 20 reveals a moderate population of positively stained (*brown*) cells throughout the spleen, with no evidence of the intense B cell populations seen in follicles or marginal zones of adult rats. CD45RA immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

the bone marrow (Fig. 4.16), and extensive diffuse hematopoietic cell proliferation (Fig. 4.17). There is a diffuse CD-3$^+$ cell population at GD 20, but no evidence of the periarteriolar lymphoid sheaths that characterize the adult spleen (Fig. 4.18).

4.2.4 Lymph Nodes

Development of the lymph node starts during embryogenesis, beginning with mesenteric lymph nodes and then proceeding to other lymph nodes (Benezech et al. 2014). Lymph nodes usually develop near large veins, typically near vein bifurcations

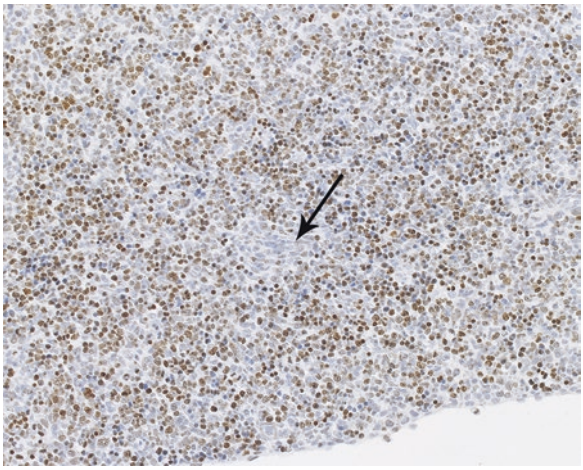

Fig. 4.17 Immunohistochemical staining for a proliferation marker performed on the spleen of a rat at GD 20 reveals intense proliferative activity throughout the spleen, except for an area immediately surrounding an arteriole (*arrow*). Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

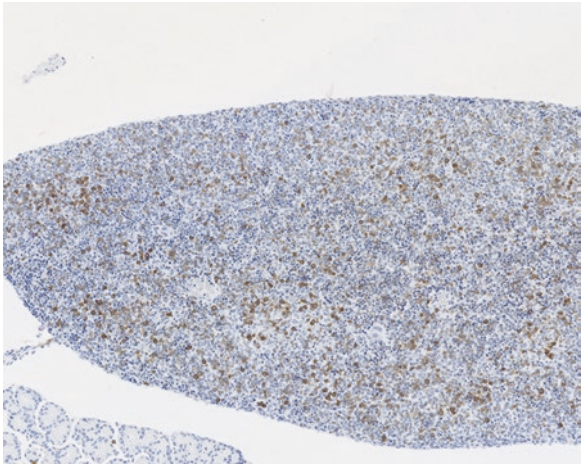

Fig. 4.18 Immunohistochemical staining performed on the spleen of a rat at GD 20 reveals a moderate diffuse population of CD3-positive cells, but no evidence of the periarteriolar lymphoid sheaths (PALS) that characterize the spleen of adult rats. CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 10× objective magnification

(Yoshida et al. 2002), which suggests the venous sites provide some type of signal that causes the localization of the emerging lymph nodes. There is recent evidence that neuronal signaling through the generation of retinoic acid may be involved in this early step in lymph node embryogenesis (van de Pavert et al. 2009).

Lymph node development in the rat occurs at a point of close approximation of a vein and a lymphatic channel. On GD 17 there is an accumulation of lymphoid cells in the mesenchyme along the wall of the vein, typically near a bifurcation of the

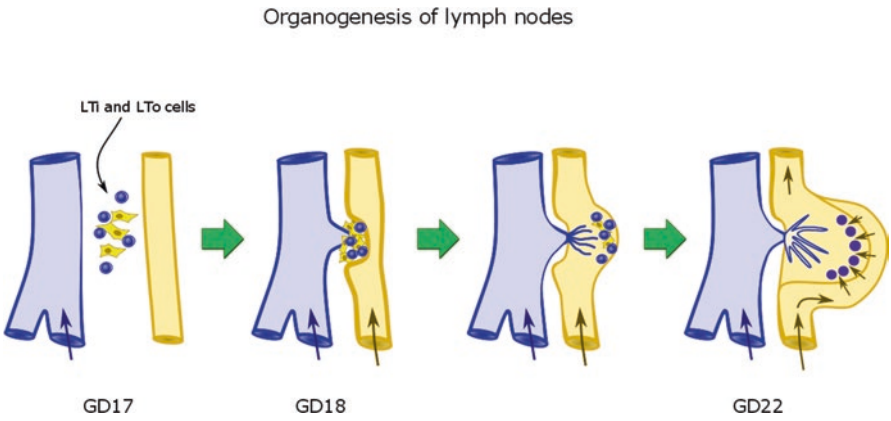

Fig. 4.19 Schematic representation of lymph node organogenesis, based on observations in the mouse. On gestation day 17 a population of lymphoid tissue initiator (LTi) cells assembles in close proximity to a lymphatic channel (*yellow*) and a vein (*blue*), typically near a bifurcation point in the vein. The LTI cells produce signaling molecules that cause nearby mesenchymal cells to assume the role of lymphoid tissue organizer (LTo) cells, which will eventually become the stromal cells of the lymph node. On GD 18 an out-pouching of the vein pushes the LTi/LTo cellular aggregation into the side of the lymphatic channel. During the next four days the out-pouching continues to encroach on the lymphatic channel until lymph flow is diverted into subcapsular sinuses of the newly formed lymph node. Direction of lymph and blood flow are shown by *arrows*

vein (Fig. 4.19) (Eikelenboom et al. 1978; Nassay and Eikelenboom 1972). The signaling processes controlling this initial location of lymph node precursors cells are largely unknown, but there is evidence the nervous system has some input into selecting the site of lymph node development (van de Pavert and Mebius 2010). The following day the lymphoid cluster forms an out-pouching that protrudes into the wall of the adjacent lymphatic channel. On subsequent days the out-pouching expands in size until it occupies the majority of the lumen of the lymphatic channel, with only subcapsular sinuses remaining to represent the original lymphatic lumen. The resultant lymph node essentially intercepts the flow of lymph through the lymphatic channel and redirects antigen- and dendritic cell-laden lymph into the subcapsular sinuses of the lymph node Development of the inguinal and popliteal lymph nodes of rabbits was similar to the histogenesis of popliteal lymph nodes in rats (Eikelenboom et al. 1978).

The model for development of lymph nodes, which originated more than a century ago (Sabin 1902), proposed that primitive lymph sacs bud from veins, and the lymph sacs are the basis for the subsequent development of lymphatics and lymph nodes. While the venous origin of lymphatics and lymph nodes remains a credible model, the requirement for lymph sacs as a prerequisite step for lymph node formation has been brought into question by the observation that primitive lymph nodes develop in E14.5 *Prox1*-null mouse embryos, which are devoid of lymph sacs and lymphatic vasculature, and in E17.5 *Prox1* conditional mutant embryos, which have defective lymph sacs (Vondenhoff et al. 2009).

The development of lymph nodes (and Peyer's patches) requires the interaction of two specialized cell populations: lymphoid tissue inducer (LTi) cells of hematopoietic origin and lymphoid tissue organizer (LTo) cells of mesenchymal origin (Lipp and Muller 2004). Embryonic LTi cells are probably derived from a population of fetal liver cells that are the counterpart of common lymphoid progenitor cells in the bone marrow of adults (Yoshida et al. 2001; Mebius et al. 2001). The LTo cells give rise to the various stromal cell populations in lymph nodes, which have multiple functions in promoting and maintaining adaptive immune responses (Cupedo et al. 2004a). The LTo cells are derived from adipocyte precursors by signaling through the lymphotoxin-β receptor (LTβR), which blocks adipocyte differentiation and promotes lymphoid stromal cell differentiation (Benezech et al. 2012). The LTo cells are critical in the formation of lymph nodes, as they provide specific signals and growth factors that facilitate movement, migration to specific areas, and maturation of LTi cells, as well as survival of lymphocytes (Roozendaal and Mebius 2011). Organogenesis assays have shown that adipocyte precursor cells of both embryos and adults can differentiate into lymph node stromal cells, thus adipose tissues continue to be a source for lymph node stroma generation in adults (Benezech et al. 2012). These interactions between adipose tissue and lymphoid structures may explain the occurrence of lymphoid tissue ('milky spots') of the omentum, fat-associated lymphoid clusters, and the common close association between lymph nodes and adipose tissue (Benezech et al. 2012; Moro et al. 2010).

Development of lymph nodes is strongly associated with inflammation-related signaling pathways. The LTi cells that first reach the site of lymph node development express lymphotoxin α1β2, a member of the tumor necrosis factor (TNF) family of inflammatory cytokines, which engages the lymphotoxin-β receptor (LTβR) on immature stromal cells and promotes maturation of those cells into lymphoid tissue organizer (LTo) cells. Signaling through LTβR induces activation of NF-κB transcription factors, leading to increased expression of chemokines and cell adhesion molecules such as ICAM-1, VCAM-1 and MadCAM-1 that are necessary for continuous recruitment and retention of LTi cells and continued development of the lymph node or Peyer's patch anlage (Randall et al. 2008; Ruddle and Akirav 2009; van de Pavert and Mebius 2010; Cupedo et al. 2004b; Ruddle 1999). Lack of LTβR signaling in genetically modified mouse models results in the absence of all lymph nodes (De Togni et al. 2014; Futterer et al. 1998; Sun et al. 2000).

Our studies (unpublished) revealed distinct lymph nodes in rats at GD 20 (Fig. 4.20), but the lymph nodes lacked the lymphocytic population, follicles and other architectural features that characterize mature lymph nodes. Following birth the lymph nodes acquire the characteristic features of cortical cellularity with follicles and germinal centers, paracortex, and medullary cords/sinuses containing various cell populations (see postnatal Sect. 4.3.4 for details).

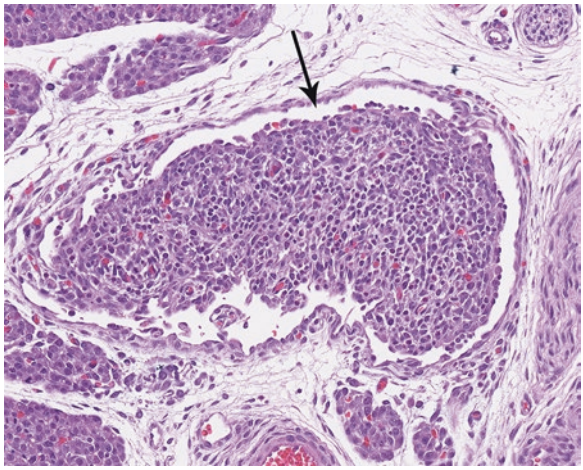

Fig. 4.20 Axillary lymph node of rat at GD 20. The immature lymph node consists primarily of stromal elements, with a minor population of lymphoid elements. The dilated subcapsular sinuses (*arrow*) indicate active fluid circulation. Hematoxylin and eosin stain, 20× objective magnification

4.2.5 Mucosa-Associated Lymphoid Tissue (MALT)

Distinct clusters of T and B cells are identifiable in the small intestine of the human fetus as early as 14–16 weeks of gestation (Cornes 1965; Kyriazis and Esterly 1971; Braegger et al. 1992). By gestation week 19 these structures are recognizable as Peyer's patches (PP), and contain follicular dendritic cells. Peyer's patches become macroscopically discernible at week 24, but germinal centers are not developed until after postnatal exposure to antigens and the intestinal microbiome (Cornes 1965).

The first stage in embryonic development of PP in mice starts at GD 14.5, when it is initiated by the expression of VCAM-1 and ligand of the tyrosine kinase receptor RET by clusters of stromal cells located on the antimesenteric side of the small intestine (Adachi et al. 1997; Veiga-Fernandes et al. 2007). During the second stage of development, between GD 14.5 and 17.5, the VCAM-1-positive cells recruit $RET^+CD11c^+cKit^+$ lymphotoxin$^+$ cells and $IL7R^+$lymphotoxin$^+$ $CD4^+CD3^-$ LTi (lymphoid tissue inducer) cells (Adachi et al. 1997; Veiga-Fernandes et al. 2007; Hashi et al. 2001). The VCAM-1^+ stromal cells express the lymphotoxin β (LTβ) receptor, and upon ligation of this receptor, the stromal cells produce IL-17 and homeostatic chemokines such as CXCL13 (Yoshida et al. 2002). This reciprocally leads to increased expression of surface lymphotoxin on LTi cells, forming a self-sustaining PP primordium (Yoshida et al. 2002; Finke et al. 2002). The third phase of PP development starts at GD17.5 when circulating lymphocytes are attracted to the T and B cell niches of the PP (Hashi et al. 2001).

Cryptopatches containing LTi cells form in the intestinal lamina propria of mice within two weeks of birth (Lugering et al. 2010), and can develop into individual lymphoid follicles (ILFs) in the presence of intestinal microflora and the lymphotoxin and CXCL13 produced by intestinal dendritic cells (McDonald et al. 2010; Tsuji et al. 2008; Hamada et al. 2002; Pabst et al. 2006; Bouskra et al. 2008; Salzman

et al. 2010). LTi-like cells are also found in secondary lymphoid tissues and the lamina propria of the intestine in adults (Cupedo et al. 2009; Tsuji et al. 2008; Scandella et al. 2008; Takatori et al. 2009). In the intestine of adults the LTi-like cells are contained primarily in cryptopatches (Eberl et al. 2004; Eberl and Littman 2004), thus supporting the concept that cryptopatches are sites of developing lymphoid structures.

Lymphotoxin and IL-7 signaling are essential for PP organogenesis during gestation in mice, but the under-developed PP seen in germ-free animals indicates a critical role for intestinal microflora during postnatal development (Neutra et al. 2001). The influence of gut microflora on PP development involves a balance between the influences of Toll-like receptors (TLRs) and gene products of the *Nod2* gene. Mice with TLR deficiency exhibit reduced size of PP, while *Nod2*$^{-/-}$ mice are characterized by hyperplasia and hypertrophy of PP (Barreau et al. 2010; Barreau et al. 2007).

Development of NALT is different from Peyer's patch development in that LTβR or RORγt signaling is not required for development of NALT, suggesting that the type of LTi cell involved in lymph node and Peyer's patch formation is not involved in NALT formation (Fukuyama et al. 2002; Harmsen et al. 2002). There is evidence that NALT formation involves a different population of LTi cells that utilize ID2 rather than LTβBR or RORγt signaling pathways (Fukuyama et al. 2002).

Our (unpublished) observations on the small intestine of rats at GD 20 revealed mucosal clusters of lymphoid cells that were consistent with developing Peyer's patches (Fig. 4.21), and early manifestations of PP were commonly present in the small intestine of rats at PND 0 (Parker et al. 2015). In our studies the lungs of rats at GD 15, GD 20, and PND0 (day of birth) were consistently devoid of bronchus-associated lymphoid tissue (BALT). Nasopharynx-associated lymphoid tissue (NALT) was absent from rats at PND 0.

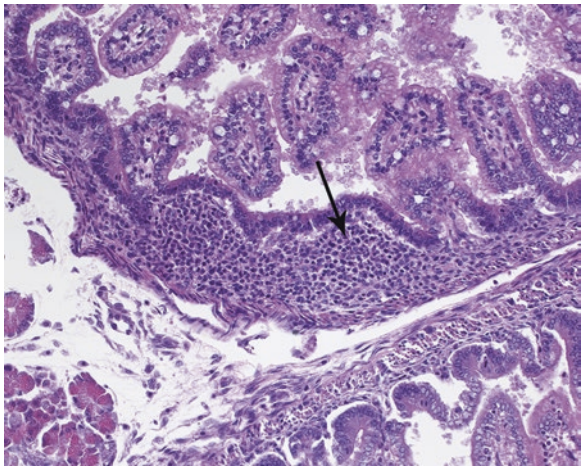

Fig. 4.21 The small intestine of a rat at GD 20 has a plaque-like aggregation of lymphoid cells (*arrow*) that represent a developing Peyer's patch. Hematoxylin and eosin stain, 20× objective magnification

4.3 Postnatal Development of Immune System Organs

4.3.1 Bone Marrow and Liver

As the bones of the rat become mineralized near the time of parturition, the medullary cavities of bones become the base for hematopoiesis. During the period from GD 20 to PND 7 there is a rapid relocation of hematopoietic activity from the liver to the bone marrow, resulting in increased bone marrow cellularity and greatly reduced hepatic hematopoiesis at PND 7. Our studies based on histological examination of postnatal rats revealed a sparse hematopoietic cell population in the bone marrow at PND 0 (Parker et al. 2015) (Fig. 4.22). At this stage the femoral marrow was slightly more cellular than the sternal marrow. At PND 7 the sternal marrow cavity remained somewhat underpopulated relative to later time points, but the femoral marrow was fully populated by hematopoietic cells. By PND 14 both the sternal and femoral marrow cavities were filled with hematopoietic cells, and were considered to be fully developed (Fig. 4.23).

At PND 0 through PND 3 the liver had a moderate diffuse hematopoietic cell population (Fig. 4.24), but by PND 10 the hepatic hematopoietic cell population consisted only of scattered clusters of cells (Fig. 4.25). A gradual decline in the level

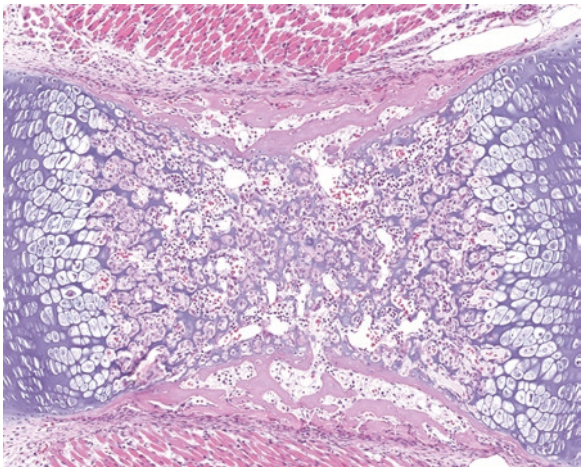

Fig. 4.22 Sternum with bone marrow of a rat at PND 0 (day of birth) has a sparse population of hematopoietic cells intermixed with partially mineralized osseous spicules. Note the thick, disorganized layer of immature bone forming the cortex of the sternebra and the dense population of immature bone spicules that extend throughout the length and width of the sternebral cavity. Hematoxylin and eosin stain, 10× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

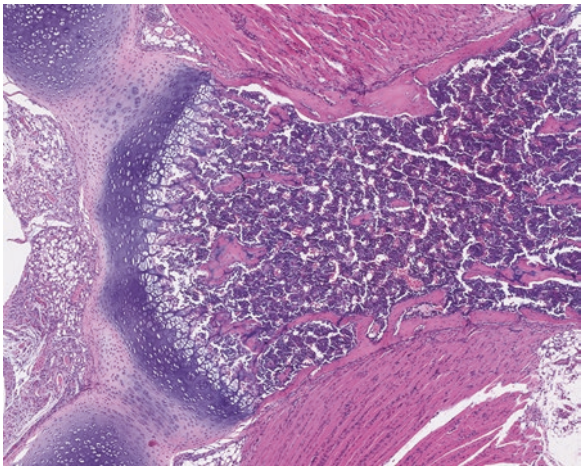

Fig. 4.23 Sternum with bone marrow of a rat at PND 14. Note the highly cellular marrow, relatively thin layer of dense cortical bone and relatively sparse (compared to Fig. 4.22) population of mature osseous spicules in the marrow cavity. Hematoxylin and eosin stain, 5× objective magnification

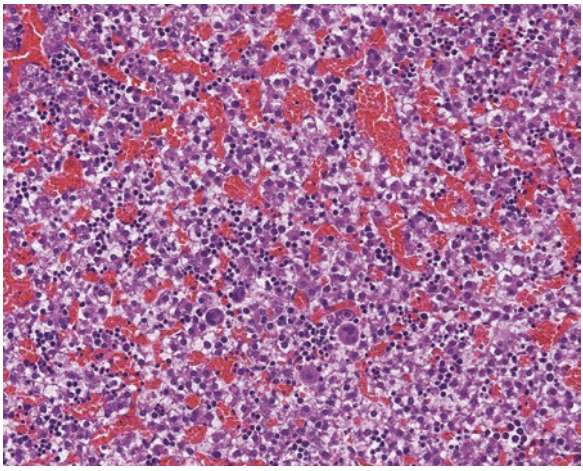

Fig. 4.24 Liver of a rat pup that was delivered by laparoscopy on PND 0 (projected day of birth, before parturition and subsequent suckling). Cords of hepatocytes throughout the section have poorly defined cytoplasmic vacuoles, consistent with glycogen accumulation. Note the moderate number of intensely basophilic hematopoietic cells within sinusoids throughout the section. Hematoxylin and eosin stain, 20× objective magnification

of hepatic hematopoiesis continued through PND 42, though widely dispersed clusters of hematopoietic cells were present in the liver of some rats on PND 42 (Fig. 4.26). Small clusters of hematopoietic cells, consisting largely of erythropoietic cells, are commonly observed in the liver of rats at the age of termination of typical 28-day repeated dose toxicology studies (i.e., age of approximately 77 days).

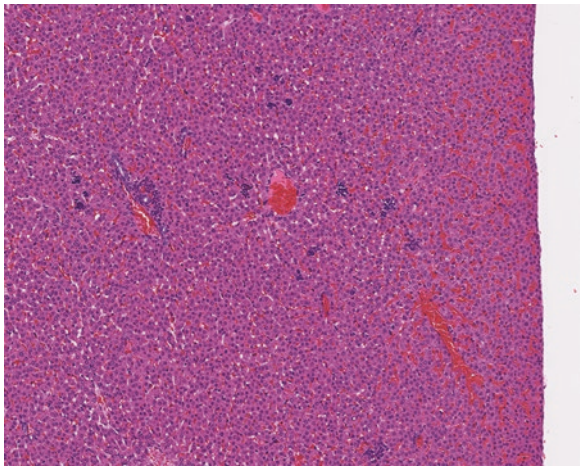

Fig. 4.25 Liver of a rat at PND 10 has clusters of deeply basophilic hematopoietic cells, but the hematopoietic activity is markedly reduced from that seen at PND 0 (Fig. 4.24). Hematoxylin and eosin stain, 10× objective magnification

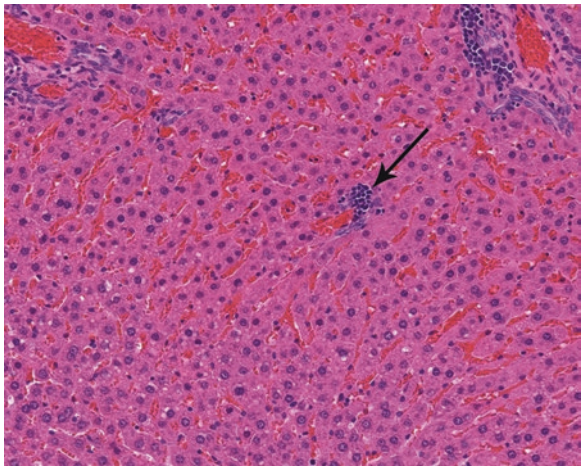

Fig. 4.26 Liver of a rat at PND 42 has small intra-sinusoidal clusters (arrow) of deeply basophilic hematopoietic cells. Hematoxylin and eosin stain, 20× objective magnification

4.3.2 Thymus

Generation of the critical peripheral pool of $CD4^+$ and $CD8^+$ T cells is most efficiently accomplished in the thymus, where interactions between immature T cell precursors and the thymic stroma favors the selection of MHC-restricted but self-non-reactive T cells (Anderson and Jenkinson 2001; Bleul et al. 2006). Thymic epithelial cells (TECs), a major component of the thymic stroma, contribute to the structural microenvironment and provide numerous developmental signals for differentiation of T cells (Anderson and Takahama 2012; Takahama 2006). The functionally distinct TEC population is divided into cortical (cTEC) and medullary (mTEC) subpopulations, which are derived from a common TEC progenitor cell population (Benz and Bleul 2005; Bleul et al. 2006). Cortical TECs positively select T cells that exhibit appropriate responses to MHC, while mTECs are involved in

negative selection of potentially autoreactive T cells (Bleul et al. 2006). Studies in mice suggest the mTEC population is derived from a previously established cTEC population, suggesting a serial progression in the development of the TEC population (Alves et al. 2014).

While there is a substantial amount of literature regarding postnatal development of immune system organs in mice and humans, information regarding postnatal development of immune system organs in rats is less abundant. In a detailed morphometric study of IHC-stained thymus and spleen of rats at 1–4, 9, 19, 23, 46 and 57 weeks of age (Kodama et al. 2012), the thymic T cell population increased markedly from three to four weeks of age, in advance of the spleen. In the reported study both the thymus and spleen exhibited mature T and B cell populations by nine weeks of age. A cytometric study of lymphoid cell development in rats throughout thymic development revealed that lymphoid cell proliferation was present in GD 14 embryos, remained high throughout embryogenesis with a peak around GD 18, decreased immediately after birth and increased again at PND 21, and then gradually decreased with age (Aboussaouira et al. 1988). Cellular proliferation in the thymus decreased even before the onset of puberty (Aboussaouira et al. 1988), suggesting the reduction in cellular proliferation was due to factors other than the onset of puberty-associated senescence. Based on cytometric analysis there are five lymphoid cell populations in the thymus of the embryonic rat: lymphoid stem cells, lymphoblasts, large lymphocytes, medium lymphocytes and small lymphocytes) (Aboussaouira et al. 1988).

The thymus undergoes a progressive remodeling that commences even before the onset of senescent involution (Quaglino et al. 1998), therefore, investigation of the postnatal development of the thymus is complicated by the simultaneous occurrence of developmental and early senescent processes that occur near the time of puberty. In a cytofluorimetric study of T cell subsets in thymus, lymph nodes and peripheral blood of rats during the first year of life, there was evidence of overlapping developmental, maturational and aging processes (Capri et al. 2000).

In a histological study of thymic development in Swiss mice at PND 1, 21, 35 and 49, the thymus was judged to have the expected components but differentiation between cortical and medullary components was not clear (Sharma et al. 2013). In the reported study the thymus was fully developed histologically at PND 35 (Sharma et al. 2013).

Our studies of the postnatal development of immune system organs in the rat indicated the thymus reaches histological maturity in advance of many other lymphoid organs, essentially in parallel with the maturing bone marrow (see Table 4.1) (Parker et al. 2015). On PND 0 the thymic cortex is thinner and somewhat less cellular than in young adult rats, though all cell populations appear to be present and corticomedullary relationships are distinct (Fig. 4.27). By PND 14 the thymus of the rat exhibits fully mature histomorphology, with highly cellular cortex and less densely cellular medulla (Fig. 4.28).

Table 4.1 Summary of compartment maturation in immune system organs of the juvenile rat based on histomorphology

	PND 0		PND 7		PND 14		PND 21		PND 28		PND 35		PND 42	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Number of animals examined	30	30	10	10	10	10	10	10	10	10	10	10	10	10
Number of litters included	5	5	6	4	8	8	8	8	9	9	10	10	9	8
Compartments/structures														
BALT														
Cellularity	0	0	0	30[a]	60	70	60	40	100	100	100	100	100	100
Follicles	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Germinal centers	0	0	0	0	0	0	0	0	0	0	0	0	0	0
High-endothelial venules	0	0	0	0	0	10	30	40	60	30	70	90	100	90
Bone marrow, femur														
Cellularity	83	92	100	100	100	100	100	100	100	100	100	100	100	100
Bone marrow, sternum														
Cellularity	25	23	100	100	100	100	100	100	100	100	100	100	100	100
GALT														
Diffuse mononuclear cells	0	0	0	0	0	0	90	100	100	100	100	100	100	100
Peyer's patches-cellularity	42	40	90	80	70	90	100	90	100	90[b]	90[c]	100	100	100
Peyer's patches-germinal centers	0	0	0	0	0	0	100	80	100	80	70	80	100	100
Lymph node, axillary														
Follicles	0	0	0	0	0	0	0	0	90	100	100	100	100	100
Germinal centers[a]	0	0	0	0	0	0	0	0	0	0	0	0	20	10
High-endothelial venules	0	0	90	80	100	90	80	90	100	100	100	100	100	100
Lymphocytes, diffuse cortical	100	88	100	100	100	100	100	100	100	100	100	100	100	100
Paracortex	0	0	30	50	90	80	100	100	100	100	100	100	100	100
Plasmacytosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymph node, mandibular														
Follicles	0	0	0	0	0	0	0	0	100	100	100	100	100	100
Germinal centers	0	0	0	0	0	0	0	0	70	80	80	90	100	100
High-endothelial venules	0	0	60	90	100	100	100	100	100	100	100	100	100	100
Lymphocytes, diffuse cortical	95	100	100	100	100	100	100	100	100	100	100	100	100	100
Paracortex	0	0	67	90	100	100	100	100	100	100	100	100	100	100
Plasmacytosis	0	0	0	0	0	0	0	0	10	20	60	80	100	100

(continued)

Table 4.1 (continued)

	PND 0		PND 7		PND 14		PND 21		PND 28		PND 35		PND 42	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Lymph node, mesenteric														
Follicles	0	0	0	0	90	100	100	100	100	100	100	100	100	100
Germinal centers	0	0	0	0	40	40	100	100	100	100	100	100	100	100
High-endothelial venules	0	0	10	90	100	100	100	100	100	100	100	100	100	100
Lymphocytes, diffuse cortical	90	90	100	100	100	100	100	100	100	100	100	100	100	100
Paracortex	0	0	90	100	100	100	100	100	100	100	100	100	100	100
Plasmacytosis	0	0	0	0	0	0	0	0	0	0	10	10	0	0
NALT														
Cellularity	0	0	10	0	0	10	30	10	30	0	90	70	60	20
Follicles	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Germinal centers	0	0	0	0	0	0	0	0	0	0	0	0	0	0
High-endothelial venules	0	0	0	0	0	10	0	0	0	0	40	50	20	0
Spleen														
Follicles	0	0	0	0	0	0	0	0	40	20	70	50	100	100
Germinal centers	0	0	0	0	0	0	0	0	0	0	60	50	100	100
Marginal zones	0	0	30	20	100	100	100	100	100	100	100	100	100	100
Periarteriolar lymphoid sheaths	59	62	100	100	100	100	100	100	100	100	100	100	100	100
Thymus														
Cortex cellularity	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Medulla cellularity	100	100	100	100	100	100	100	100	100	100	100	100	100	100

[a]Percentage of rats in each group that had histologic evidence the compartment or structure was present

[b]Peyer's patches not present in recut sections at multiple levels

[c]Peyer's patches not present in recut sections at multiple levels. Individual lymphoid follicles present

Shading indicates the compartment or structure was present in the indicated percentage of rats but the overall cellularity of the compartment or prominence of the structure in some rats was less than that seen at later time points

From: Parker et al. (2015). Histologic features of postnatal development of immune system organs in the Sprague-Dawley rat. *Toxicologic Pathology* 43: 794–815

In the early stages of development the thymus sometimes exhibits a less densely cellular subcapsular zone that contains a substantial population of large mononuclear cells, consistent with the 'large lymphocyte population' in the thymic marginal zone described in the embryology literature (Harland 1940), and the population of lymphoblasts and/or large lymphocytes described in cell proliferation analysis of the developing thymus (Aboussaouira et al. 1988). The only subsequent change through PND 42 is an increase in the overall size of the organ.

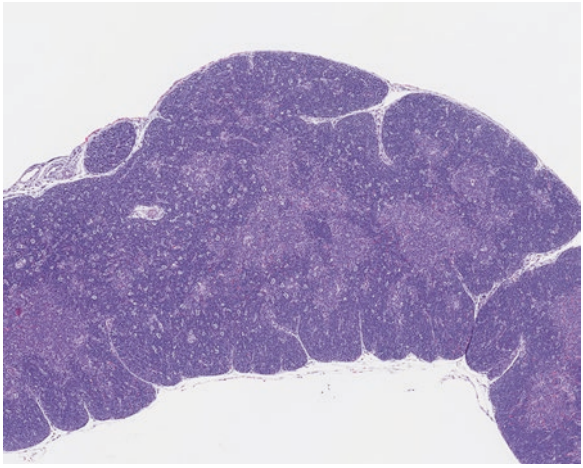

Fig. 4.27 The thymus of rat at PND 0 (day of birth) has distinct corticomedullary differentiation, with *dark-stained cortical regions* consisting of dense populations of lymphocytes and less intensely stained medullary regions. The relative cross-sectional area of cortex relative to medulla is less than that seen in the thymus of adult rats (i.e., reduced corticomedullary ratio). Hematoxylin and eosin stain, 2.5× objective magnification

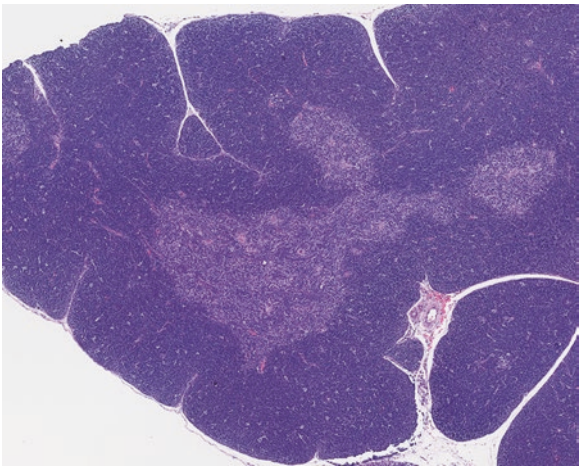

Fig. 4.28 The thymus of a rat at PND 14 has increased cortical thickness, relative to the thymus at PND 0. The thymus is larger than at PND 0 (note different image magnifications), and it continues to enlarge somewhat as the juvenile animal increases in size. Hematoxylin and eosin stain, 1.25× objective magnification

4.3.3 Spleen

Literature regarding postnatal development of the spleen in rats is sparse. A morphometric study of the spleen of rats revealed the area and cellularity of the white pulp increased postnatally until nine weeks of age and remained stable thereafter (Kodama et al. 2012). Lymphoid follicles were not apparent histologically until three weeks of age, and were clearly visible at four weeks of age. T cell populations in PALS and marginal zones increased with age up to nine weeks and remained

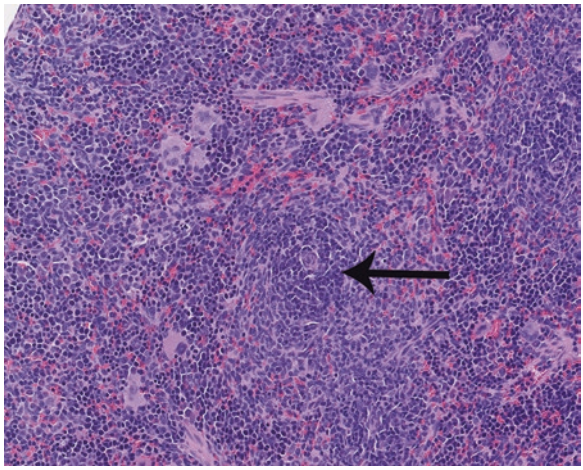

Fig. 4.29 The spleen of a rat at PND 0 (day of birth) has a periarteriolar (*arrow*) aggregate of lymphoid cells, but has no evidence of lymphoid follicles, germinal centers or marginal zone. The surrounding red pulp has a mixture of hemolymphopoietic cells. Hematoxylin and eosin stain, 20× objective magnification

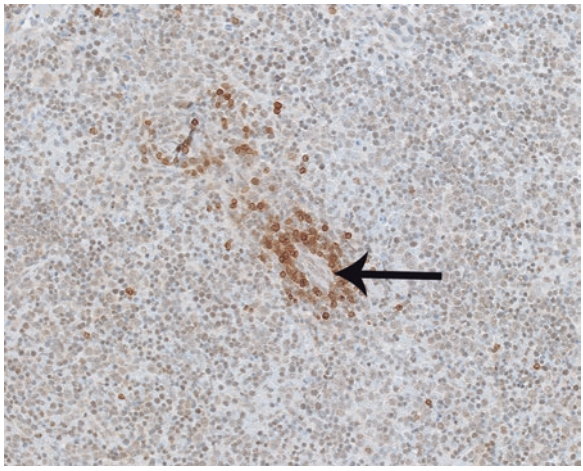

Fig. 4.30 A population of brown-stained $CD3^+$ cells, consistent with T cells or T cell precursors, surrounds an arteriole (*arrow*) in the spleen of a rat at PND 0 (day of birth). CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

stable thereafter. B cell populations increased with age in all areas, with the most pronounced populations contained in lymphoid follicles.

Our studies in rats (Parker et al. 2015) indicated the postnatal histological development of the splenic immune system components is similar to that observed in mice (Kaufman and Bard 1999; Landreth 1993; Paige et al. 1981). At PND 0 there are aggregations of $CD3^+$ mononuclear cells surrounding splenic arterioles (Figs. 4.29 and 4.30), consistent with an early manifestation of periarteriolar lymphoid sheaths (PALS). There are no lymphoid follicles, germinal centers, or other evidence of B cell-dependent structures at PND 0. On PND 7 there is expansion of the periarteriolar cellular sheath, which typically has a peripheral zone of large mononuclear cells and less pronounced, centrally placed population of mature lymphocytes. From PND 14 through PND 28 the PALS become more prominent and

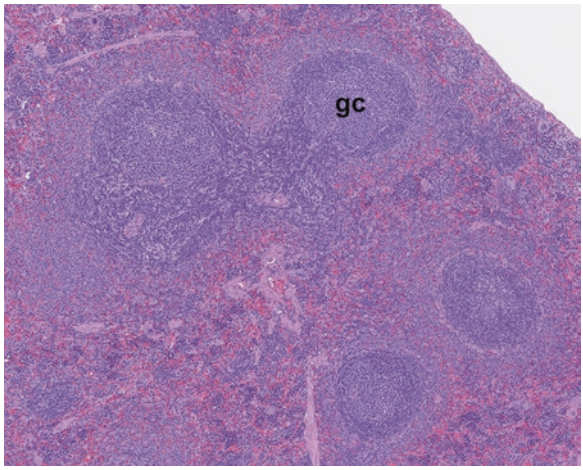

Fig. 4.31 The fully developed spleen of a rat at PND 42 has numerous follicles with germinal centers (*gc*), consistent with secondary follicles that have developed in response to antigenic stimulation. Hematoxylin and eosin stain, 2.5× objective magnification

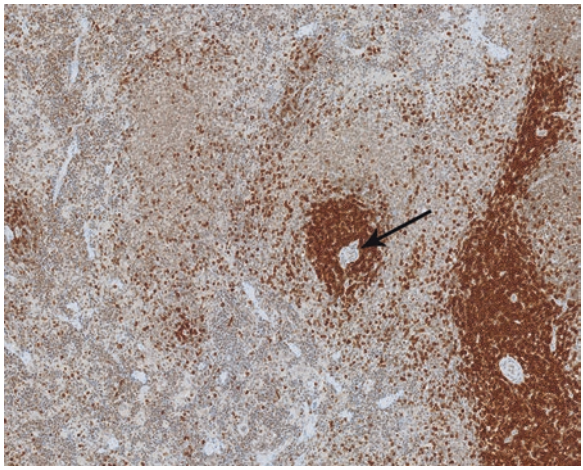

Fig. 4.32 Spleen of rat at PND 42 showing brown-stained CD3-positive cells around arterioles (*arrow*). CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 10× objective magnification

cellular, but follicles and germinal centers have not developed. On PND 35 there are well-formed follicles with germinal centers in a small percentage of animals, and on PND 42 these structures are uniformly present (Fig. 4.31). Immunohistochemical (IHC) staining for CD3 and CD45RA on rat spleens collected at PND 42 reveals prominent T cell and B cell differentiation (Figs. 4.32 and 4.33), respectively, and IHC staining for Ki67 (Fig. 4.34) indicates substantial proliferative activity in germinal centers of secondary lymphoid follicles (Parker et al. 2015).

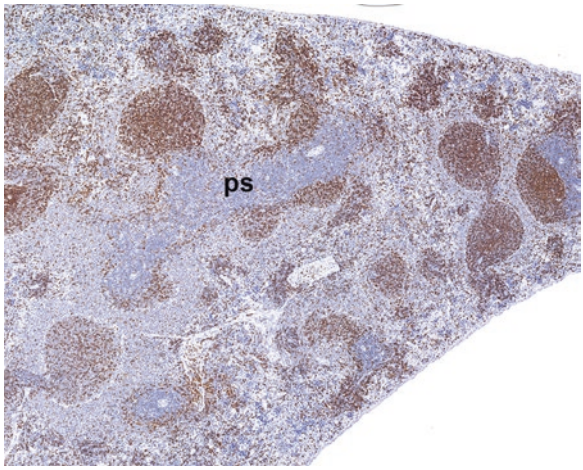

Fig. 4.33 Spleen of rat at PND 42 showing brown-stained CD45RA-positive cells in follicles. Note sparse CD45RA staining in periarteriolar lymphoid sheath (*ps*). CD45RA immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 5× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

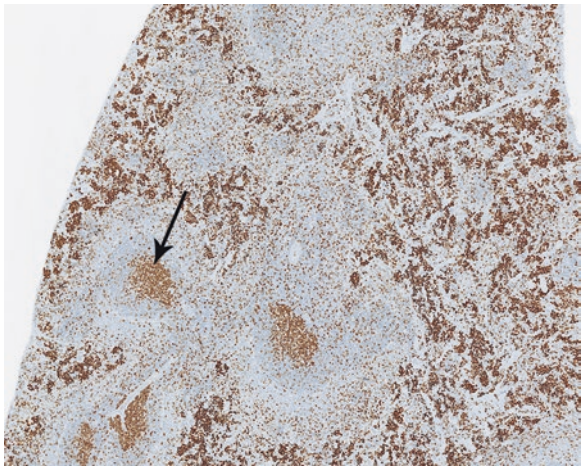

Fig. 4.34 Spleen of rat at PND 42 showing proliferative activity (brown-stained nuclei) diffusely in red pulp and in germinal centers (*arrow*). Note substantial proliferative activity in the hematopoietic cell population in the red pulp. Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 5× objective magnification

4.3.4 Lymph Nodes

Lymph nodes develop relatively late in fetal life and persist throughout adulthood (Losco and Harlemen 1992). Their histologic appearance at any given time is determined by the stage of postnatal development or senescence, and the type and magnitude of antigenic stimulation (Tizard 1987). Lymph nodes of neonatal mice have a distinct reticular framework with a small number of lymphocytes in the cortex (Arno 1980). Immunohistochemical studies of popliteal lymph nodes of immature rats revealed T cells and interdigitating cells (dendritic cells) were present from birth (van Rees et al. 1985). B cells were present in the lymph nodes of neonatal mice on the second day of life, and the first primary follicles appeared at day 18

(van Rees et al. 1985). When subjected to strong immunizing signals such as injections of TNP-KLH in the hind footpads in young rats, at five days following immunization the popliteal lymph nodes had primary follicles when immunization occurred at 19 days of age and secondary follicles with germinal centers when immunization occurred at 23 days of age (van Rees et al. 1986). A similar study of responses to thymus-independent type-1 (TI-type 1) and TI type-2 antigens (trinitrophenyl-lipopolysaccharide, (TNP-LPS) and TNP-Ficoll, respectively) revealed a specific antibody response to TNP-LPS with immunization at 19 days of age, but antibody response to TNP-Ficoll was low even when immunization occurred at six weeks after birth (van Rees et al. 1987). The latter observations highlight the potential disconnection between morphologic evidence of maturity in immune system organs versus full functional maturity with ability to react to all types of antigens.

Observations in our studies follicles (Parker et al. 2015) indicated that postnatal development of lymph nodes in rats occurs somewhat later than the thymus and bone marrow, and there are differences in the postnatal developmental schedule of enteric (e.g., mesenteric and mandibular) versus somatic (e.g., axillary) lymph nodes. On PND 0 the axillary, mandibular and mesenteric lymph nodes are poorly developed histologically, consisting of stromal elements and vascular structures with minimal lymphocytic populations (Fig. 4.35). At this stage of postnatal development the lymph nodes have no follicles, germinal centers, paracortex, or high-endothelial venules, and there is no evidence of plasma cell accumulation in medullary sinuses. This is followed by sequential development of the various lymph node compartments, with development of the T cell compartment (paracortex) and evidence of immunoreactivity (prominent high-endothelial venules) preceding development of the B cell compartments (follicles, germinal centers and plasma cell populations) (see Table 4.1). Histologic development of the mesenteric lymph nodes occurs somewhat before development of the mandibular lymph nodes, and well before development of the axillary lymph nodes. Mesenteric lymph nodes of rats are

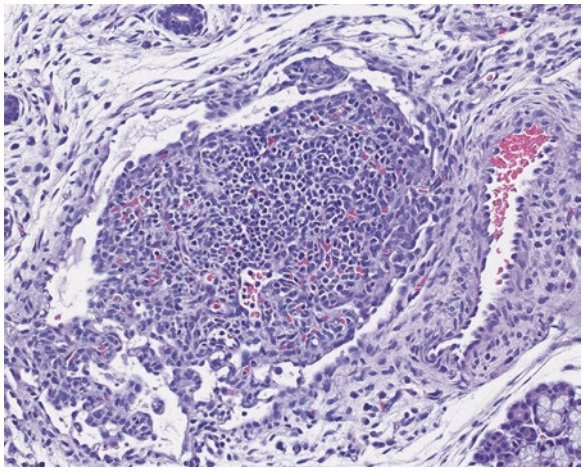

Fig. 4.35 The mandibular lymph node of a rat at PND 0 (day of birth) consists primarily of stromal elements, with a sparse population of deeply stained lymphocytes. Hematoxylin and eosin stain, 20× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

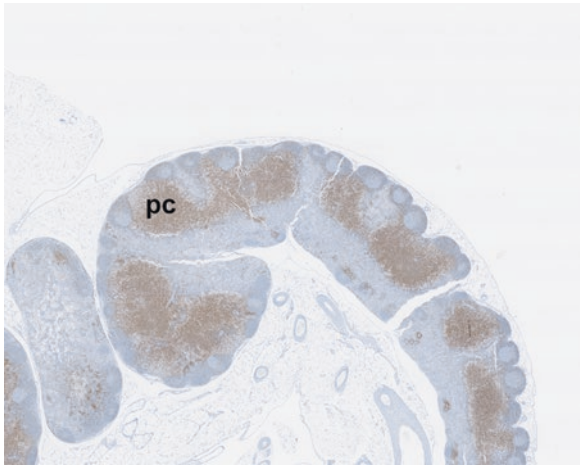

Fig. 4.36 Mesenteric lymph node from rat at PND 42 showing T cell population in paracortex (*pc*). CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 1.25× objective magnification

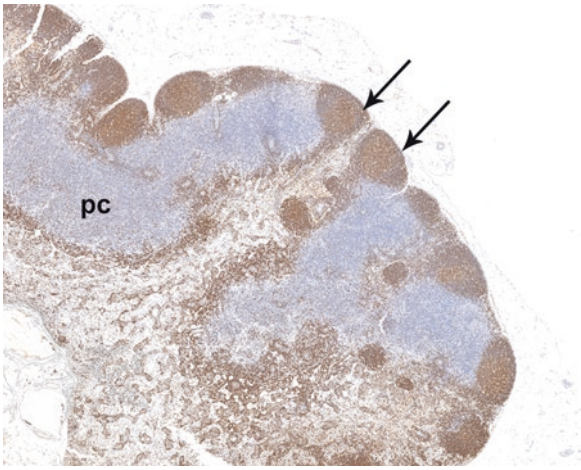

Fig. 4.37 Mesenteric lymph node from the same PND 42 rat shown in Fig. 4.36, showing prominent B cell follicles (*arrows*) in cortex and distinct paracortical zone (*pc*). Note CD45RA-positive cells in medullary cords. CD45RA immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 2.5× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

completely developed on PND 21, as evidenced by the presence of paracortex with high-endothelial venules and secondary follicles with germinal centers (Figs. 4.36–4.38). By contrast, the axillary or mandibular lymph nodes have no follicles or germinal centers, which suggests the B cell-dependent zones of those lymph nodes are not fully developed at this stage. Histologic development of the B cell-dependent compartments of the mandibular lymph nodes follow soon after development of the mesenteric lymph nodes, but axillary lymph nodes do not typically have secondary follicles with germinal centers by PND 42.

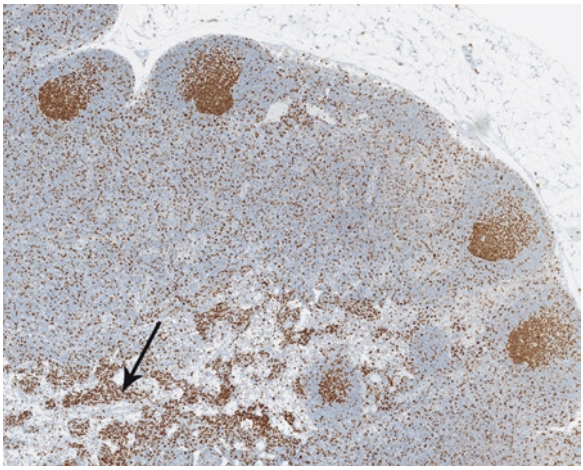

Fig. 4.38 Mesenteric lymph node from the same PND 42 rat shown in Figs. 4.37 and 4.38, showing brown-stained cells indicating proliferative activity in germinal centers of four secondary lymphoid follicles. Note the scattered population of proliferative cells throughout the cortex, and concentration of proliferative cells in medullary cords (*arrow*). Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 2.5× objective magnification

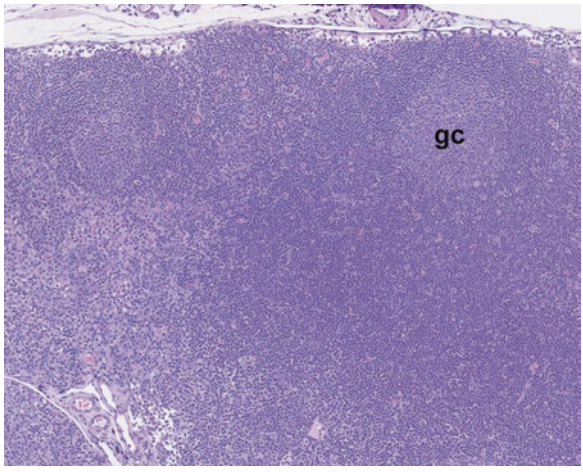

Fig. 4.39 By PND 35 the mandibular lymph node of a rat is fully developed histologically, with highly cellular paracortex and numerous secondary follicles containing lightly stained germinal centers (*gc*). Hematoxylin and eosin stain, 5× objective magnification

In addition to differences in postnatal development, the axillary, mandibular and mesenteric lymph nodes of rats have consistent differences in histomorphology. Medullary cords of the mandibular lymph nodes have more pronounced populations of plasma cells than are seen in mesenteric or axillary lymph nodes (Figs. 4.39 and 4.40). Axillary lymph nodes rarely have germinal centers at PND 42. The chain of

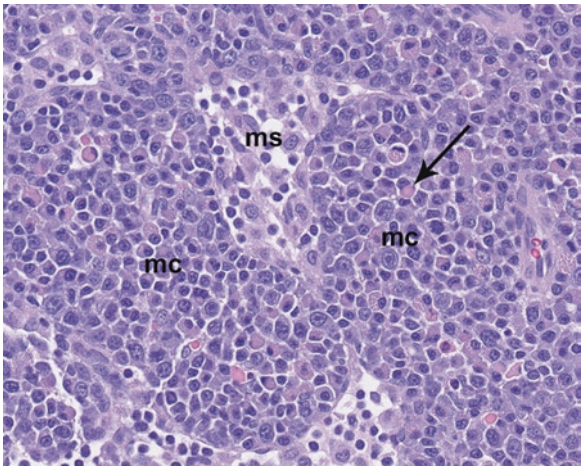

Fig. 4.40 Higher magnification of the mandibular lymph node shown in Fig. 4.39 has two medullary cords (*mc*) containing numerous plasma cells that are characterized by eccentrically placed nuclei and paranuclear lightly stained areas that represents the Golgi apparatus. Mature plasma cells engorged with eosinophilic cytoplasmic immunoglobulin-rich material (*arrow*) are known as Mott cells. The intervening medullary sinus (*ms*) contains small, mature lymphocytes and a few large histiocytic cells. Hematoxylin and eosin stain, 40× objective magnification

individual lymphoid follicles that constitute the elongated mesenteric lymph nodes commonly exhibit different stages of histologic development in different regions of the lymph node, presumably reflecting different levels of immunologic stimulation from various drainage areas. These observations are a reminder that what is recognized as full histologic development of lymph nodes is partially dependent on the level of environmental stimulation. The environmental contribution to the histologic appearance of lymph nodes may be altered if the immunostimulatory input is reduced, e.g., following administration of test articles that alter the intestinal microbiome.

4.3.5 Mucosa-Associated Lymphoid Tissue (MALT)

MALT is named according the tissue in which it occurs, e.g. GALT for gut-associated lymphoid tissue, BALT for bronchus-associated lymphoid tissue, and NALT for nasopharynx-associated lymphoid tissue (Brandtzaeg et al. 2008). MALT in any location has common functional and structural features that support capture of antigens from an overlying epithelial surface and presentation of those antigens to the underlying cellular elements of the immune system. However, MALT in different tissues has some somewhat variable development, structure and function.

4.3.5.1 Gut-Associated Lymphoid Tissue (GALT)

Gut-associated lymphoid tissue consists of Peyer's patches, individual lymphoid follicles (ILF), lymphocyte-filled villi, and cryptopatches in the small intestine, diffuse lymphocytic populations in the mucosa of the small and large intestine, and submucosal lymphoid aggregates (lymphoglandular complexes) in the large intestine.

GALT formation commences in late gestation and extends into the postnatal period in most species, but is completed during gestation in sheep and some additional ruminant animals (Reynolds and Morris 1983). An immunohistochemical and histological study (Chen et al. 1995) of PP formation in rats revealed surface-IgM (sIgM)-bearing cells (B cells) scattered throughout the PP at PND 1. On PND 3, sIgM-bearing cells accumulated to form primary follicles that were associated with domed elevations in the surface epithelial contour. Cells that were immunohistochemically positive for the OX-2 marker (follicular dendritic cells-FDC) were present in the center of primary follicles by PND 4. Germinal centers first appeared within the lymphoid follicles of PP on PND 18, and by PND 21 most follicles contained a germinal center. PP that formed soon after birth were smaller and had fewer follicles than those formed later in the postnatal period. On PND 5 each PP contained 6–8 lymphoid follicles, with the lymphoid follicles subsequently increasing in size and number. The mean number of follicles/PP reached the adult level (11.1 follicles/PP) on PND 21 and remained at that level for the next 15 weeks, though individual follicles continued to enlarge during the observation period. The relative distribution of lymphocyte sub-types in PP at four weeks after birth was similar to that of mature rats, but the histomorphology of the tissue was not considered well differentiated until a week later.

A study based on fluorescence microscopy of labelled cells extracted from the small intestine of neonatal and juvenile rats revealed shifts in the PP cell populations as the organs matured during the early postnatal period (Lyscom and Brueton 1983). The numbers and relative proportions of lymphocyte sub-types were not mature at the time of weaning (approximately three weeks of age), but were mature at four weeks after birth. However, the histomorphology of the tissue was not considered to be fully developed until a week later.

Peyer's patches and the diffuse intestinal lymphocyte population have different postnatal development sequences and somewhat different immunobiological features, but share responsibility for production of IgA (Beagley and Elson 1992), which is a critical component of defense against enteric pathogens (Endt et al. 2010; Hooper and Macpherson 2010). In human neonates and infants the gastrointestinal immune system is functionally immature, and a full level of intestinal IgA production does not occur until two years of age (Savilahti 1972). Studies in rats have shown the IEL population increases gradually from birth and reaches a mature level at six weeks of age (Lyscom and Brueton 1983). A transient increase in IEL population in rats at postnatal week one is suspected to represent an influx maternal lymphocytes from the colostrum ('milk lymphocytes') (Lyscom and Brueton 1983). This maternal lymphocyte population is also known to accumulate in the colostrum

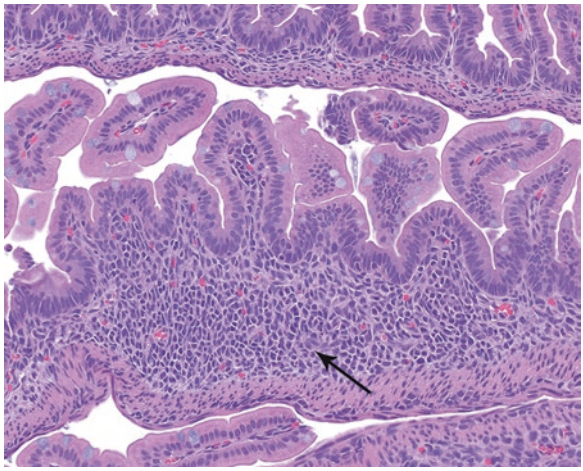

Fig. 4.41 The small intestine of a rat at PND 0 (day of birth) has an immature Peyer's patch (*arrow*) consisting of a plaque-like aggregation of lymphoid cells. Hematoxylin and eosin stain, 20× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

of humans (Diaz-Jouanen and Williams 1974; Richie et al. 1982), and has been shown to cross the intestinal mucosal barrier in rats (Seelig and Billingham 1981). The survival of ingested maternal lymphocytes is aided by the absence of significant gastric acid in neonatal rats. Gastrin, the hormone responsible for stimulating HCl production by the parietal cells, is not produced in sufficient quantities and gastrin receptors on parietal cells are insufficient for full physiological function until PND 18 (Henning 1981; Walthall et al. 2005); therefore, HCl production does not occur to a significant degree until PND 18 in the rat, and the low pH level of the adult stomach is not achieved until PND 40 (Walthall et al. 2005). Absence of the sterilizing effect of gastric acid also allows establishment of the intestinal microbiome, which in turn aids in development of MALT and establishment of mucosal immunity (Pabst et al. 2006).

There are subtle differences in the terminology applied to the diffuse lymphoid cell population in the small intestine, and these differences may result in apparent conflicts in numerical data related to mucosal cell populations in the intestine. In some instances the term 'intraepithelial lymphocytes' is restricted to those lymphoid cells that are located within the superficial epithelial layer of the intestinal mucosa. In other instances the term is expanded to include lymphoid cells in the lamina propria of the intestinal mucosa. In our studies (Parker et al. 2015) the gut-associated lymphoid tissue (GALT) of the small intestine was scored histologically as two separate elements: mucosal lymphoid follicles (Peyer's patches-PP) and diffuse mucosal mononuclear cell population, the latter representing a combination of intraepithelial lymphocytes (IEL) and the mononuclear cell population in the lamina propria. On PND 0 the small intestinal mucosa has mucosal cellular aggregates that are consistent with developing PP (Fig. 4.41), but the mucosa is essentially devoid of diffuse mononuclear cell populations (Fig. 4.42). The developing Peyer's patches become progressively larger and more cellular through PND 14 (Fig. 4.43) and PND 21. They do not contain germinal centers at PND 14 (Fig. 4.44). Germinal

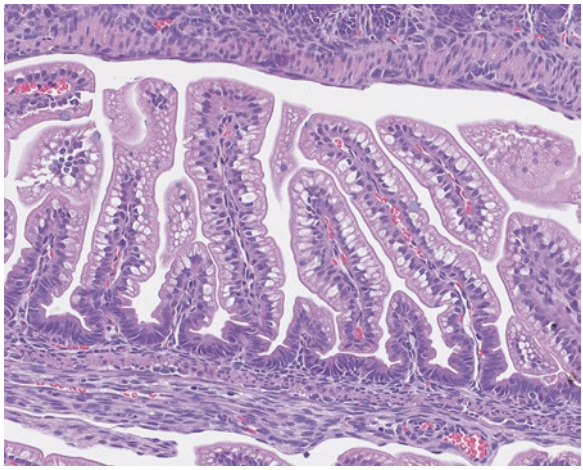

Fig. 4.42 The small intestine of a rat at PND 0 (day of birth) is essentially devoid of a diffuse lymphocytic population. Clear vacuoles and lightly eosinophilic globules in epithelial cells represent absorbed nutrients subsequent to nursing. Hematoxylin and eosin stain, 20× objective magnification

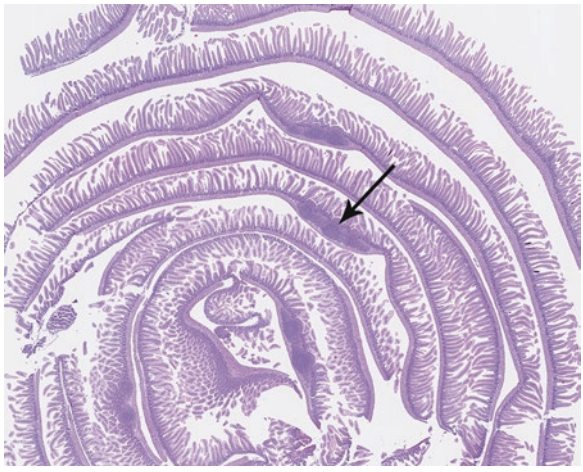

Fig. 4.43 Swiss roll preparation of small intestine of rat at PND 14 shows multiple Peyer's patches (*arrow*). The ileum is at the center of the Swiss roll. Exact boundaries between duodenum, jejunum and ileum are difficult to determine in rats. Hematoxylin and eosin stain, 1.25× objective magnification

centers first appeared in PP at PND 21 (Fig. 4.45), but the PP were not considered to be fully developed until PND 28 (Figs. 4.46 and 4.47). On PND 28 the small intestinal mucosa had additional components of GALT such as individual lymphoid follicles (ILF) (Fig. 4.48), distinct lymphocyte-filled villi (Fig. 4.49), and cryptopatches (CP) (Fig. 4.50), the latter being randomly distributed clusters of lymphoid cells in the basilar lamina propria (Pabst et al. 2005). Structures with morphologic features intermediate between cryptopatches and individual lymphoid follicles were noted (Fig. 4.51), suggesting cryptopatches may be predecessors of individual lymphoid follicles. Development of the diffuse intestinal mononuclear cell population lagged behind the development of PP, and was not fully developed until PND 35 (Fig. 4.52).

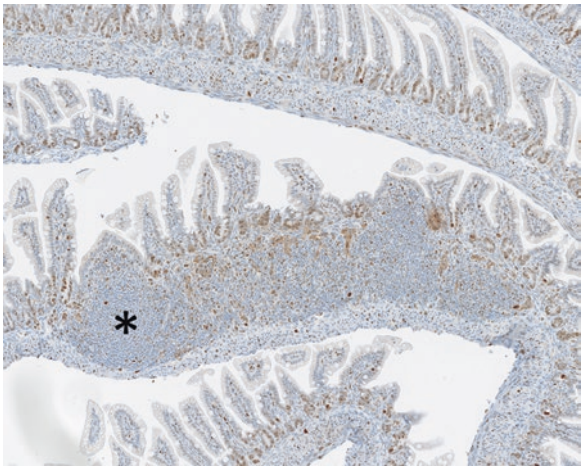

Fig. 4.44 Immunohistochemical staining reveals no proliferative cells in the Peyer's patch follicles (*asterisk*) at PND 14. Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

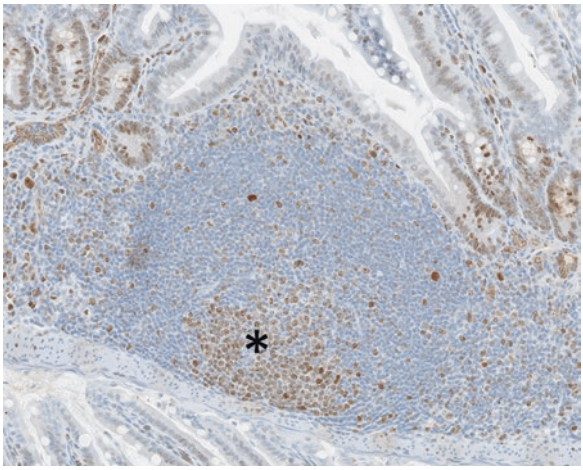

Fig. 4.45 Immunohistochemical staining reveals proliferative cells in the germinal center (*asterisk*) at the base of the Peyer's patch follicles at PND 21. Ki67 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 10× objective magnification

Information related to PP of the small intestine should not be directly extrapolated to the mucosal lymphoid follicles (lymphoglandular complexes) of the large intestine, as these structures have some anatomical and functional differences (Crouse et al. 1989; Perry and Sharp 1988; Owen et al. 1991). There are similarities between the structures, e.g., in the processing of enteric antigens for presentation to lymphoid elements, but in some ways the lymphoglandular complexes of the large

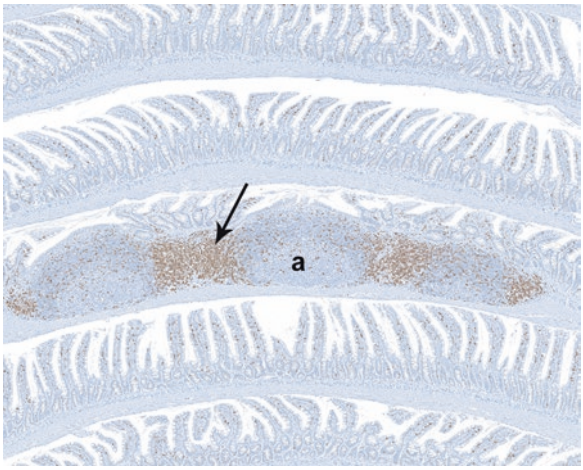

Fig. 4.46 Small intestine with Peyer's patch from PND 28 rat showing T cell areas (*arrow*) interspersed between follicles (*a*). Note widely scattered population of CD3-positive cells in mucosal epithelium. CD3 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 5× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

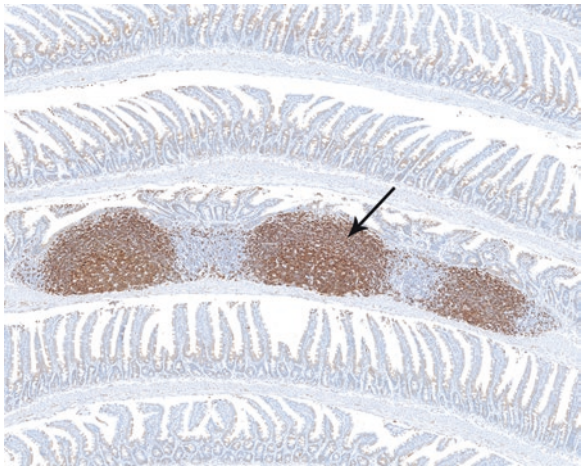

Fig. 4.47 Small intestine with Peyer's patch from PND 28 rat showing prominent B-cell follicles (*arrow*). CD45RA immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain, 5× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

intestine more nearly resemble the thymus than Peyer's patches, particularly in the response to corticosteroid administration (Crouse et al. 1989). Our studies have indicated that mucosal lymphoid aggregates are present in the large intestine of the rat as early as PND 7 (Fig. 4.53), and are well-developed by PND 21 (Fig. 4.54).

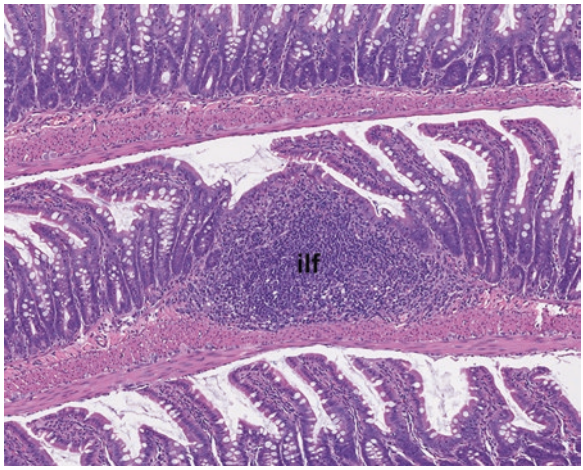

Fig. 4.48 The small intestinal mucosa of a rat at PND 28 has an individual lymphoid follicle (*ilf*). Note the dome-shaped elevation of the overlying follicle-associated epithelium, which is similar to that seen in Peyer's patches. Also note the extended base of the structure resembles a cryptopatch (see Fig. 4.50). Hematoxylin and eosin stain, 10× objective magnification

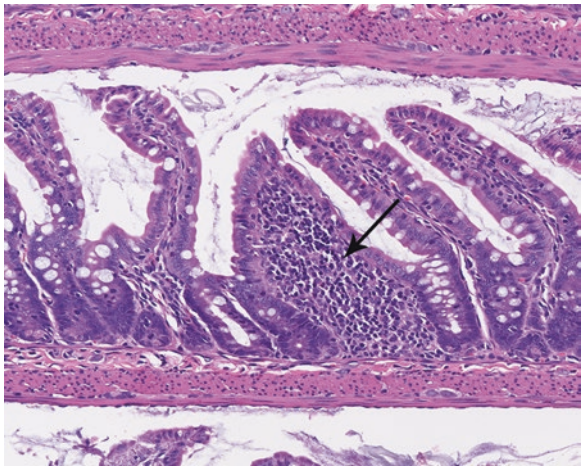

Fig. 4.49 Small intestine of rat at PND 28 with lymphocyte-filled villus (*arrow*). Note the lymphoid cell population is contained entirely within the villus, with no involvement of the underlying submucosa. Hematoxylin and eosin stain, 20× objective magnification

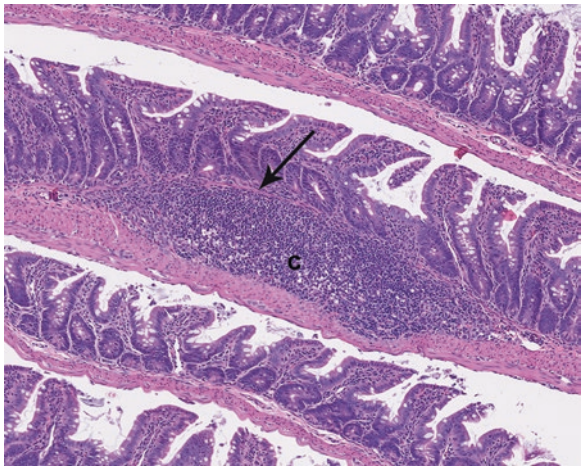

Fig. 4.50 Small intestine of a rat at PND 28 with cryptopatch (*c*). Note the structure is located deep to the mucosa and muscularis mucosa (*arrow*). Hematoxylin and eosin stain, 10× objective magnification

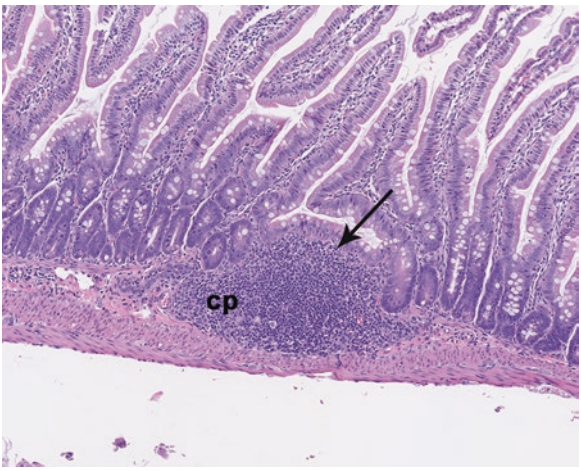

Fig. 4.51 Small intestine of a rat at PND 28 with a cryptopatch (*CP*) showing features of transition into an isolated lymphoid follicle. Note the intense, follicle-like cellularity in the center of the CP and the slight dome-shaped elevation in the overlying epithelium (*arrow*). Hematoxylin and eosin stain, 10× objective magnification

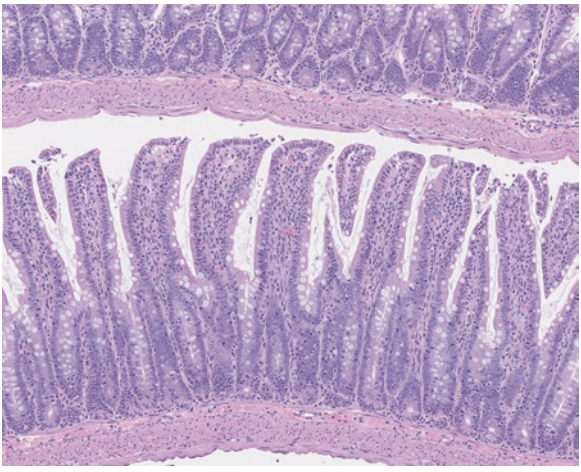

Fig. 4.52 Small intestine of a rat at PND 35 has a moderate number of lymphoid cells in the lamina propria of villi, with less lymphoid cellularity in the deeper aspects of the mucosa. Hematoxylin and eosin stain, 20× objective magnification

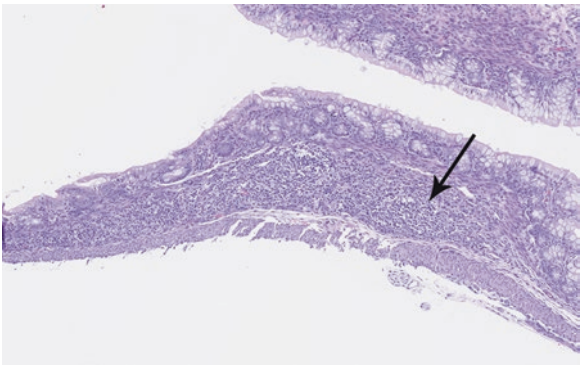

Fig. 4.53 Rectum of a rat at PND 7 with an immature submucosal lymphoid aggregate (*arrow*). Hematoxylin and eosin stain, 10× objective magnification. (Reproduced from Parker (2016) Chapter 10: Immune System. In: Parker, G. A. and Picut, C. A. (eds.) Atlas of histology of the juvenile rat, Academic Press/Elsevier

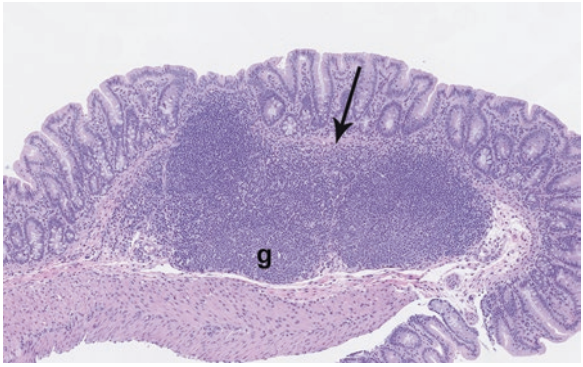

Fig. 4.54 Cecum of a rat at PND 21 with a submucosal lymphoid structure that contains multiple germinal centers (*g*). Note the lymphoid structure is located primarily beneath the muscularis mucosa (*arrow*), but breaches the muscularis mucosa in two areas. In contrast to the highly attenuated follicle-associated epithelium (FAE) of Peyer's patches in the small intestine, there is no histologically discernible alteration of the overlying intestinal epithelium in this large intestinal structure. Hematoxylin and eosin stain, 10× objective. (Reproduced from Parker (2016) Chapter 10: Immune system. In: Parker, G. A., Picut, C. A. (eds.) Atlas of histology of the juvenile rat, Academic Press/Elsevier

4.3.5.2 Bronchus-Associated Lymphoid Tissue (BALT)

Bronchus-associated lymphoid tissue (BALT) is a constitutively developed secondary lymphoid tissue in rats and rabbits, but is present in mice and humans only when associated with inflammation in the lungs ('iBALT'). Bronchus-associated lymphoid tissue was not present in rats of our studies (Parker et al. 2015) at PND 0, but by PND 7 minor peribronchiolar cellular aggregates consistent with developing BALT were occasionally noted (Fig. 4.55). By PND 14 and 21 an increased number of rats had histologically distinct BALT, and at PND 28 through 42 BALT was present to some degree in all rats. As early as PND 14 the BALT of some rats had distinct high-endothelial venules (HEV), and these structures remained clearly visible as BALT cellularity increased through PND 35 and PND 42 (Fig. 4.56). BALT varied in cellularity and overall prominence in individual animals. Distinct follicles and germinal centers were not seen in BALT of rats through PND 42.

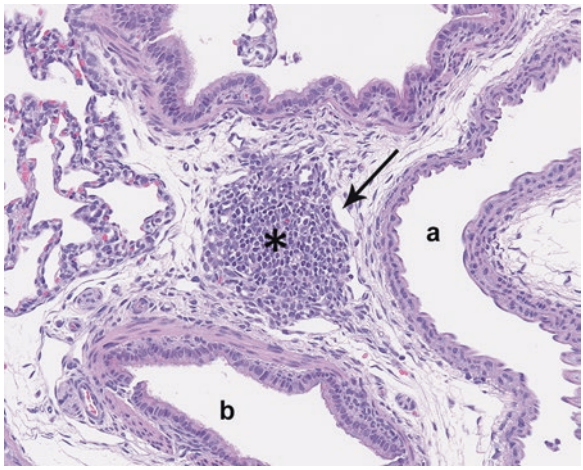

Fig. 4.55 Lung of a rat at PND 7 with developing bronchus-associated lymphoid tissue (BALT) (*asterisk*) located near arteriole (*a*) and bronchiole (*b*). Note lymphatics (*arrow*) near the BALT. Hematoxylin and eosin stain, 20× objective magnification. (Reproduced from Parker et al. (2015), *Toxicologic Pathology* 43: 794–814)

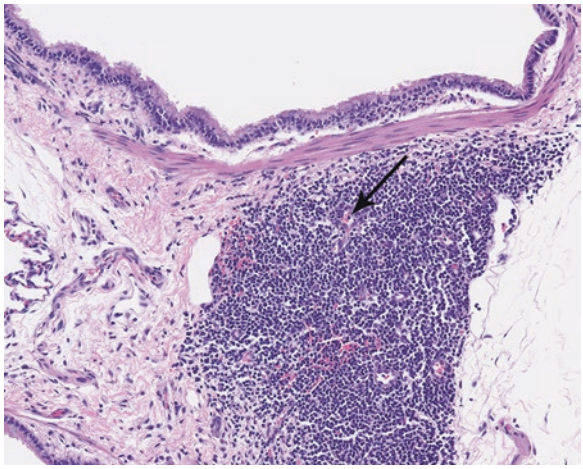

Fig. 4.56 Lung of a rat at PND 42 with fully developed bronchus-associated lymphoid tissue (BALT). Note high-endothelial venules (*arrow*) in BALT. Hematoxylin and eosin stain, 20× objective magnification

4.3.5.3 Nasopharynx-Associated Lymphoid Tissue (NALT)

Formerly known as nasal-associated or nose-associated lymphoid tissue (NALT), the currently accepted terminology for the lymphoid structures in the posterior aspect of the nasal cavity of rats is 'nasopharynx-associated lymphoid tissue' (Brandtzaeg et al. 2008). These aggregations of lymphoid tissue in the nasopharynx of rats are similar to tonsils and adenoids (Waldeyer's ring) in humans (Ruddle and Akirav 2009).

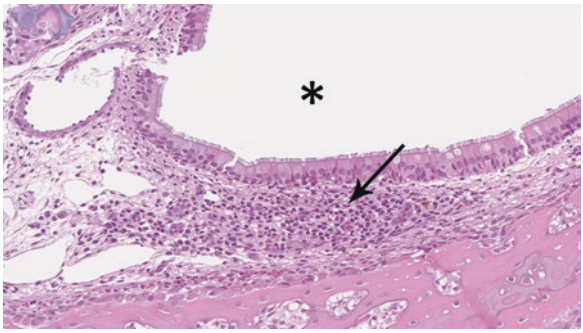

Fig. 4.57 Posterior aspect of nasal cavity of a rat at PND 7 showing nasopharynx-associated lymphoid tissue (NALT) (*arrow*) in the ventrolateral aspect of the nasopharynx (*asterisk*). Hematoxylin and eosin stain, 5× objective magnification. Hematoxylin and eosin stain, 20× objective magnification. (Reproduced from Parker (2016) Chapter 10: Immune system. In: Parker GA, Picut CA (eds) Atlas of histology of the juvenile rat. Academic Press/Elsevier

Despite similarities in structure and function, there are significant differences in the organogenesis and postnatal development of PP versus NALT. The HEV of PP express mucosal vascular addressin cell-adhesion molecule (MAdCAM-1) (Csencsits et al. 1999), while NALT-associated HEVs express peripheral-node addressin (PNAd) (Kiyono and Fukuyama 2004). As a result, different populations of naïve B cells are sequestered into PP versus NALT. Precursors to PP were observed in mice as early as GD 14.5 (Adachi et al. 1997), and dome-shaped PP were observed in later stages of embryogenesis in mice (Hashi et al. 2001). By contrast, NALT was not observed during embryogenesis or in newborn mice (Fukuyama et al. 2002). High-endothelial venules with associated $PNAd^+$ lymphocytes were first detected in nasal tissue of mice at PND 7, but the complete histologic structure of NALT was not present until five to eight weeks after birth (Fukuyama et al. 2002). These observations in mice suggest prenatal initiation of lymphoid organogenesis for Peyer's patches and postnatal organogenesis for NALT (Kiyono and Fukuyama 2004). Based on observed PNAd expression of lymphocytes in NALT, the nasal lymphoid tissue apparently attracts the same population of lymphocytes that is attracted to lymph nodes. Sparse published observations on rats suggest NALT development in rats is similar to that seen in mice (Hameleers et al. 1989).

In our study of the development of immune system organs follicles (Parker et al. 2015), the nasopharyngeal region of rats at PND 0 had no lymphoid elements. Minor mucosal lymphocytic clusters were first noted in the basolateral aspect of the nasopharynx at PND 7 (Fig. 4.57), and were sporadically present at remaining time points. High-endothelial venules were variably present within the nasopharngeal lymphoid aggregates, but neither follicles nor germinal centers were observed in NALT at any time up to PND 42. These observations suggest the presence of NALT is highly variable, presumably influenced by environmental stimulation.

4.4 Tertiary Lymphoid Tissue and Milky Spots

The tertiary (ectopic) lymphoid tissues that form at sites of chronic inflammation have many features in common with secondary lymphoid organs (Aloisi and Pujol-Borrell 2006; Drayton et al. 2006; Carragher et al. 2008; Cupedo and Mebius 2005), and their genesis apparently results from interactions of the same signaling molecules as are involved in the embryogenesis of secondary lymphoid organs (Aloisi and Pujol-Borrell 2006; van de Pavert and Mebius 2010). In such situations the pro-inflammatory cytokine profile exists in the 'fertile soil' of fatty tissue that contains precursor cells which are capable of becoming LTo cells. The only remaining requirement is 'seeding' by LTi cells from the bone marrow, and tertiary lymphoid follicles may be formed as a result. The tertiary lymphoid tissue may have a positive effect in promoting immune responses (Aloisi and Pujol-Borrell 2006), or may have negative effects through the generation of self-destructive autoantibodies (Penaranda et al. 2010; Lee et al. 2006) or inappropriate induction of tolerance to antigens such as tumor-associated antigens (Shields et al. 2010).

Milky spots in the omentum, which contain primarily B-1 cells and macrophages, develop independently of LTi cells and lymphotoxin-α signaling (Rangel-Moreno et al. 2007), therefore probably have a developmental path that is different from the other lymphoid organs (van de Pavert and Mebius 2010).

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Chapter 5
Aging of Immune System Organs

George A. Parker

Abstract Aging of mammals has a negative effect on virtually all components of the immune system. Some, but not all, age-related immunological alterations are due to structural alterations in immune system organs or depletion of immunocyte populations. However, other age-related alterations involve dysregulation and failures in coordination of various immunological functions. Aging effects on the innate immune system may result in a pro-inflammatory status ('inflamm-aging') that consists of a paradoxical increase in the tendency to inflammation, but concurrently reduced ability to mount an effective inflammatory response. Many of the age-related alterations in adaptive immune responsiveness are a result of a decline in T cell function, which has a downstream effect on B cell functions and humoral immune responses.

Keywords Immune system • Histology • Aging • Senescence • Lymph nodes • Thymus • Spleen • Bone marrow • Immunobiology

Abbreviations

APC	antigen-presenting cell
ARR	antigen-responsive reticulum
BALT	bronchus-associated lymphoid tissue
BLA	basic lead acetate
BTLA	B and T lymphocyte attenuator
CCR	chemokine receptor
CD	clusters of differentiation
CINC	cytokine-induced neutrophil chemoattractant
CLP	Common lymphocyte progenitor
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DC	dentritic cell
DHEA	dehydroxyepiandosterone

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DHEAS	dehydroepiandrosterone sulfate
DTH	delayed type hypersensitivity
Fcγ	fraction crystallizable γ
FDC	follicular dendritic cell
GALT	gut-associated lymphoid tissue
G-CSF	granulocyte colony-stimulating factor
HSC	Hematopoietic stem cell
IFNγ	Interferon γ
IL-	Interleukin
MALT	mucosa-associated lymphoid tissue
MC	mast cell
MHC	major histocompatibility class
MIP	macrophage inflammatory protein
MOCD	monocyte-origin dentritic cells
NALT	nasopharynx-associated lymphoid tissue
NET	neutrophil extracellular trap
NK	Natural killer
NKT	NK cell with T cell receptor
PD-L1	programmed death ligand 1
PECAM	platelet-endothelial cell adhesion molecule
PI3K	phosphoinositide 3 kinase
RANTES	regulated on activation normal T cell expressed and secreted
TCR	T cell receptor
T_{FH}	T follicular helper cell
TFN-α	Tumor necrosis factor-α
T_{FR}	T follicular regulatory cell
TLR	Toll-like receptor
TREM-1	triggering receptor expressed on myeloid cells-1

5.1 Introduction

Aging has a deleterious effect on many mammalian organ systems, and the immune system is no exception. Many effects of aging on the immune system are imperceptible unless specific assays are employed, while other effects are clearly apparent. The thymus starts to involute near the time of puberty and is markedly involuted by late adulthood. The bone marrow undergoes a progressive age-related reduction in cellularity, with replacement of hematopoietic cells by adipocytes. Secondary lymphoid organs such as spleen and lymph nodes retain their basic histomorphology despite aging, but have subtle evidence of altered immune responsiveness. More definitive studies reveal a plethora of age-related changes that affect virtually all cells and organs of the immune system. Many of these changes involve a decrement in cell numbers or functions, but some involve a more complex dysregulation of immune system functions. This chapter provides

an overview of age-related alterations in the various components of the immune system, and provides examples of age-related change in immune system organs of animal species that are commonly employed in safety assessment toxicology studies.

5.2 General Features of Immunosenescence

Aged humans have greater morbidity and mortality from infectious disease, which is believed to be in part due to age-related changes in immune function. The effects of the associated chronic disease processes have a major impact on world-wide human health, thus are a significant problem confronting humanity (Dietert et al. 2012). Aging is accompanied by a generalized reduction or dysregulation in immune system function, commonly referred to as 'immunosenescence'. Immunosenescence affects both innate and adaptive arms of the immune system (Allman and Miller 2005b; Gomez et al. 2005; Weng 2006), but effects of aging on the adaptive immune system are more pronounced (Weiskopf et al. 2009). Immunosenescence affects various cell types in the bone marrow and thymus, mature lymphocytes in the peripheral blood and secondary immune system organs, and elements of the innate immune system (Weiskopf et al. 2009). Common effects of aging include decreased T cell memory, decline of naïve T cell populations (Weng 2006); decline in B cell production with defective humoral immunity (Allman and Miller 2005a) and a chronic inflammatory state ('inflamm-aging') (Franceschi et al. 2000). Subclinical inflammation may be caused by the partial inability of the aged immune system to eliminate certain pathogens, or chronic stimulation of the immune system by the signaling and effector molecules associated with inflammation (inflamm-aging) (Franceschi et al. 2000). The subclinical inflammatory status in turn contributes to the development of organ-specific age-related diseases such as osteoporosis, neurodegeneration and atherosclerosis (Libby et al. 2002; Libby 2002; Gao and Hong 2008; Ginaldi et al. 2005).

As in humans, immune senescence in nonhuman primates is characterized by chronic inflammation and increased susceptibility to infections and neoplasms (Didier et al. 2016). Adaptive immune responses in rhesus macaques are more affected by aging than innate immune responses (Haberthur et al. 2010).

5.3 Effects of Aging on Hematopoietic Stem Cells and Committed Lymphoid Progenitor Cells

Cells of the immune system are derived from hematopoietic stem cells (HSCs), which proliferate and commit to erythroid, lymphoid, or myeloid lineages. The total amount of bone marrow hematopoietic tissue in humans decreases with age (Hartsock et al. 1965), and there is an age-related decrease in the capacity for renewal of hematopoietic stem cells (HSCs) (Lansdorp et al. 1994). The ability of

human HSCs to proliferate correlates inversely with age (Geiger 2002), possibly due to shortening of telomeres (Vaziri et al. 1994). Age-related changes do not affect erythroid and myeloid progenitors (Weiskopf et al. 2009), but with increasing age there is skewing toward the myeloid lineage at the expense of the lymphoid lineage, which is due to clonal expansion of myeloid-biased HSCs (Beerman et al. 2010). Studies have demonstrated that bone marrow or purified HSCs from old mice have reduced ability to generate lymphoid cells (Tyan 1977; Hirokawa et al. 1986; Sharp et al. 1990; Sudo et al. 2000), resulting in a substantial reduction in the population of common lymphocyte progenitors (CLP) in mice by 7 months of age (Miller and Allman 2003). In addition to direct effects of aging, age-related changes in hormone production may disturb self-renewal and lineage commitment of HSCs (Dykstra and de Haan 2008; Chen 2004; Linton and Dorshkind 2004).

Aging has specific effects on bone marrow production of cells involved in the adaptive immune system. Aging results in production of fewer pro-B cells in the marrow and a smaller number of these cells undergo subsequent differentiation, resulting in overall lower production of mature B cells (Cancro 2005). Bone marrow-derived T cell precursors are less affected by aging than B cell precursors but, due to an age-related decrement in T cell maturation in the thymus, the peripheral T cell compartment is also substantially reduced with age (Weiskopf et al. 2009). In addition to the declines in absolute cell numbers, the lymphoid cell populations of aged subjects also undergo shifts in function that serve to further reduce or dysregulate immune function, as discussed below.

5.4 Effects of Aging on the Innate Immune System

The innate immune system is the first barrier to invasion of the mammalian body by external pathogens. The skin, which may be considered the largest organ of the 'immune system', constitutes a physical barrier to invasion by microbial pathogens. The epithelial lining of the gastrointestinal, respiratory, urinary, and reproductive tracts have a similar barrier function, as do the epithelial surfaces of the conjunctiva and ear canal. Studies have shown that aging is frequently associated with increased permeability of epithelial barriers of the skin, lung, or gastrointestinal tract (Weiskopf et al. 2009), which allows invasion of pathogenic organisms that present additional challenges to the aging adaptive immune system (Gomez et al. 2005; Ershler and Keller 2000).

In additional to anatomical barriers, physiological barriers are critically important in nonspecific immune function. The low pH of gastric contents serves to sterilize ingesta, thus reducing the potential for invasion by microbial pathogens. The flow of inspired air past various obstacles of the nasal passages and airways introduces turbulence that causes inhaled particles to be thrown against mucus-covered epithelial surface, where they are trapped and expelled. The mucociliary clearance mechanisms of the trachea and lung capture potential pathogens and physically transport them out of the respiratory system. Intestinal motility continuously propels potential pathogens through the intestinal tract to allow elimination in the feces.

Interruption of intestinal motility, e.g., in post-surgical paralytic ileus, can have disastrous consequences. The physical and physiological barrier function of the intestine is aided by actions and products of the adaptive immune system, including massive production of IgA that binds microbes into clusters in the intestinal lumen, thus impeding their motility and invasive ability. Other physiological mechanisms that contribute the innate immunity are less apparent, such as ureteral peristalsis that aids in urine flow (Osman et al. 2009), thus preventing urine stasis that can lead to ascending bacterial infections, or the flow of tears that eliminates irritants and potential pathogens from the surface of the eye.

In addition to these anatomical and physiological components of the innate immune system, there are multiple categories of molecules that serve to repel, disable, or destroy potential pathogens. These molecules are considered at greater length in Volume 1, Chap. 3.

Cellular elements of the innate immune system, including neutrophils, NK and NKT cells, dendritic cells, and monocytes/macrophages, serve as backup protection against infection when anatomic and/or physiologic barriers fail or are overwhelmed. There is evidence that aging affects all cells of the innate immune system (Agrawal et al. 2008; Gomez et al. 2005; Plackett et al. 2004; Plowden et al. 2004), and that age-associated defects are commonly linked to compromised signal transduction pathways such as the Toll-like Receptors (TLRs). In addition to altered signaling pathways, aging is associated with decreased phagocytic activity of macrophages and dendritic cells, reduced natural killer (NK) cell cytotoxicity, and dysregulated production of immunoregulatory molecules such as cytokines and chemokines (Weiskopf et al. 2009).

While many aspects of the innate immune system are diminished with aging, there is some evidence of age-related hyper-reactivity in innate immune responses (Kovacs et al. 2009; Panda et al. 2009; Gomez et al. 2008a; Gomez et al. 2009). As a general concept, immunosenescence of the innate immune system appears to represent dysregulation rather than simple impairment of immune function. The dysregulation results in a pro-inflammatory environment with persistent low-grade immune activation that may augment damage caused by infections and degenerative diseases in the elderly (Dietert et al. 2012). Age-related increases in plasma levels of interleukin-6 (IL-6), IL-1β, and TNFα are reported in elderly populations, and may be predictive markers of functional disability, frailty, and mortality (Ershler and Keller 2000; Bruunsgaard 2002; O'Mahony et al. 1998). Studies involving intracellular cytokine staining in splenocytes from rhesus monkeys have also shown an age-related increase in the frequency of inflammation-associated IFNγ$^+$ and TNF-α$^+$ T cells, particularly the CD8$^+$ effector memory T cell population (Jankovic et al. 2003), suggesting nonhuman primates have age-related immunological deficits similar to those observed in humans.

While it is clear the immune system suffers various forms of age-related degradation and dysregulation, consideration must also be given to 'non-immune' contributors to generalized immune function. The immune system does not exist in isolation, and is continuously modulated by a variety of signaling molecules such as hormones (Shaw et al. 2010). Aging is associated with alterations in these endocrine functions, which directly influence immune functions. For example, in humans the serum level of dehydroepiandrosterone sulfate (DHEAS) at age 70 is only 10–20%

of the level seen at age 30, while cortisol level remains nearly constant (Shaw et al. 2010). Dehydroepiandrosterone sulfate and its successor molecule (dehydroxy-epiandosterone-DHEA) have immuno-enhancing effects (Hazeldine et al. 2010). Decline in these endocrine factors may result in immunological risk in old age, particularly in aged males, as stress may result in increased immunosuppressive cortisol levels but the counter-balancing immuno-enhancing effect of DHEAS/DHEA is not present due to age-related decline in those elements (Radford et al. 2010; Butcher et al. 2005). As a result, the immunosuppressive effects of cortisol predominate over the immunoprotective influence of androgens following stress in aged males. Similar hormonal influences are present in laboratory animal species. For example, there is an age-related decrement in multiple leukocyte functions of rats, and it has been shown that ovariectomy reduces the immunologic advantage that is typically enjoyed by aged females (De la Fuente et al. 2004).

5.4.1 Effects of Aging on Neutrophils

When discussing the effects of aging on neutrophils and other circulating cells, a distinction must be made between aged cells, i.e., those cells that have been in the circulation for a prolonged period of time, versus cells of aged subjects, which refers to cellular populations of unspecified individual cell age that were derived from aged subjects.

Neutrophils constitute the primary defense against rapidly dividing bacteria, yeast and fungi, and are a critical part of the acute inflammatory reaction. Neutrophils in the circulating blood are recruited to sites of infection by chemokines and products released from microorganisms (Chilvers et al. 2000; Davis et al. 1987; Lehrer et al. 1988b). Neutrophils extravasate from blood vessels near sites of inflammation via sequential adhesion to low-affinity selectin molecules followed by binding to high-affinity integrin molecules (Cooper et al. 1995; Doyle et al. 1997), and trans-migration through the wall of post-capillary venules is facilitated by platelet-endothelial cell adhesion molecule (PECAM-CD31) (Muller et al. 1993).

Neutrophil precursors in the bone marrow and circulating neutrophil numbers are well-preserved in aged humans, but progenitor cells from aged humans have reduced proliferative capability in response to granulocyte colony-stimulating factor (G-CSF) stimulation (Chatta et al. 1993). The initial process of neutrophil emigration to sites of inflammation seems to be unaffected by aging (Butcher et al. 2000). However, there is a significant decrement in the ability of neutrophils from aged individuals to phagocytize opsonized bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Wenisch et al. 2000). The decrement in phagocytosis by neutrophils of aged individuals is associated with reduced expression of Fc receptors (CD16) (Butcher et al. 2001), which normally serve to bind opsonizing immunoglobulin molecules to the surface of neutrophils and facilitate phagocytosis of the immunoglobulin/microbe complexes. By contrast, there is no age-associated difference in phagocytosis of non-opsonized bacteria (Emanuelli et al. 1986), suggesting that receptors for the innate recognition of bacteria (e.g. the LPS receptor CD14) are not affected by aging.

Following phagocytosis of microbes into neutrophils, microbial killing is accomplished by generation of reactive oxygen and nitrogen species as well as release of toxic enzymes and microbicidal peptides contained in cytoplasmic granules of neutrophils (Lehrer et al. 1988a; Wessels et al. 2010). Neutrophils of aged subjects have a generalized decrease in phagocytic capacity and intracellular killing (Fulop et al. 2004; Tortorella et al. 2007). A number of age-related decrements in downstream signaling and effector pathways render neutrophils less capable of performing these functions (Wessels et al. 2010). Neutrophils from aged humans have reduced superoxide generation (Miyaji et al. 2000) and neutrophils from 24-month-old rats have reduced superoxide burst in response to experimental stimulation (Shaw et al. 2010). Superoxide generation and phagocytosis mediated by Fcγ receptor on neutrophils are decreased in elderly humans (Lloberas and Celada 2002). Recruitment of Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1) to lipid rafts in cell membranes is reduced in neutrophils from elderly donors, resulting in impairment of phagocytosis, respiratory burst and degranulation in neutrophils (Fortin et al. 2007). This may explain the reduction in levels of lipopolysaccharide-induced TLR4 in lipid raft fractions of neutrophils from aged donors (Fulop et al. 2004), as TREM-1 is known to be involved in TLR signaling (Shaw et al. 2010).

Effete neutrophils, i.e., aged individual circulating neutrophils, normally return to the bone marrow via the process of chemotaxis (Weisel et al. 2009), thus impairment of chemotactic ability in neutrophils from aged subjects serves to increase the circulating population of effete or semi-effete neutrophils that are known to have reduced phagocytic ability as well as reduced superoxide burst that is critical to neutrophil-mediated microbial killing (Shaw et al. 2010). This age-related impairment in neutrophilic chemotaxis reduces the rate of return of aged neutrophils to the bone marrow, where the somewhat effete but nevertheless dangerous neutrophils can be safely dismantled. Accumulation of semi-effete, chemotactically impaired neutrophils in peripheral tissues of aged subjects increases the potential for bystander tissue injury as neutrophils release proteases to aid migration through tissues (Nomellini et al. 2008a; Nomellini et al. 2008b).

In addition to the well-known processes of phagocytosis and intracellular killing by multiple mechanisms, neutrophils also kill extracellular microbes by releasing Neutrophil Extracellular Traps (NETs) (Brinkmann et al. 2004). Activated neutrophils release granule proteins and chromatin that together form extracellular fibers (NETs) that bind Gram-positive and -negative bacteria, prevent the organisms from spreading, and ensure a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria (Brinkmann et al. 2004). Generation of NETs is reduced in neonates versus adults (Yost et al. 2009), and is known to decline in aged humans (Hazeldine et al. 2014).

In summary, aging of the host may result in an increase in the circulating population of aged, semi-effete neutrophils, with an attendant potential for damage to host tissue due to augmented inflammatory processes, yet the individual circulating neutrophils have reduced ability to perform their normal protective functions against microbial pathogens.

5.4.2 *Effects of Aging on Monocytes and Macrophages*

Monocytes represent a mobile component of the innate immune system which respond to inflammation by differentiating into antigen-presenting cells such as macrophages and dendritic cells (Shaw et al. 2010). Macrophages play an important role in both the innate and adaptive immune systems, thus age-related changes in macrophages may impact both arms of immune responsiveness (Weiskopf et al. 2009). In the innate immune system, macrophages serve in the initiation of nonspecific inflammatory responses, direct elimination of pathogens, regulation of the innate system, and removal of the products of inflammation and tissue damage. In the adaptive immune system, macrophages serve a regulatory function and serve as 'professional antigen-presenting cells' (APCs) that process and present antigenic peptides to T cells in the context of MHC class II molecules.

The bone marrow of aged humans has a reduced population of macrophages and macrophages precursors (Plowden et al. 2004). Somewhat paradoxically, aged humans have an increased number of circulating monocytes that is nevertheless correlated with increased overall clinical frailty (Della Bella et al. 2007; Leng et al. 2009). Investigations of this apparent paradox have revealed there is an age-related decrease in macrophage function, particularly in monocyte/macrophage-mediated Toll-Like Receptor (TLR) activation, even though the absolute number of circulating monocytes is increased with aging (Shaw et al. 2010).

Aging influences multiple macrophage functions, including phagocytic activity, cytokine and chemokine secretion, antibacterial defenses, infiltration and wound repair, and antigen presentation (Sebastian et al. 2005). Studies in rats have shown a 75% reduction in ability of macrophages from aged animals to produce superoxide anion (Plackett et al. 2004), which is an integral part of the respiratory burst that occurs as macrophages destroy phagocytized microbes. This reduction in intracellular killing of microbes may result in prolonged duration of infections (Plackett et al. 2004), thus contributing to the pro-inflammatory milieu of the aged ('inflamm-aging').

The collaboration between the innate and specific immune systems is demonstrated in a number of macrophage activities, some of which are altered with aging. As the phagocytic ability of macrophages declines with age, there is a parallel age-related reduction in the levels of macrophage-derived chemokines such as MIP-1α, MIP-1β, MIP-2, and eotaxin (Swift et al. 2001) that extends the impact of the macrophage deficit to multiple other immunological processes. Some of these age-related monocyte/macrophage deficits have far-reaching consequences:

- Age-related reduction in TLR activation in monocyte/macrophage populations results in lower expression of CD80, a costimulatory signal that is necessary for T cell activation and survival (Shaw et al. 2010). Up-regulation of CD80 expression upon TLR engagement is important in the generation of protective antibody response to vaccination, thus this age-related decline in monocyte/macrophage function may hinder the induction of clinically desirable adaptive immunity (Shaw et al. 2010).
- Macrophages from aging humans and rodents have reduced expression the MHC class II molecules that are required for presentation of antigenic peptides to $CD4^+$

T cells (Zissel et al. 1999; Plowden et al. 2004; Herrero et al. 2002), thus aging of macrophages may contribute to a generalized reduction in the $CD4^+$ helper T cell responses that are critical to generation of effective humoral immunity.

- Defects in monocyte/macrophage function have also been demonstrated in delayed-type hypersensitivity (DTH) responses to *Candida* antigens in aged humans (Agius et al. 2009). This latter observation has obvious implications with regard to immunity and clinical condition in aged humans, but also raises questions regarding the reliability of DTH-based clinical tests such as tuberculin skin testing in aged individuals (Shaw et al. 2010).

Age-associated alterations in macrophage numbers and function are also seen in tissue-adapted macrophages such as Kupffer cells of the liver, which may have important implications for systemic immune responses as well as hepatic function. A study of young (4–6 months), middle-aged (13–14 months), and old (24–26 months) male Fischer 344 rats revealed an age-related increase in the number of Kupffer cells as well as an age-related increase in the rate of clearance of polystyrene microspheres, the latter indicating increased phagocytic capacity (Hilmer et al. 2007). In contrast, livers from aged (22 months) mice exhibited significant decreases in the uptake of carbon and in the carbon-induced O_2 consumption compared to young (3 months) animals, with a concomitant diminution in the carbon-induced O_2 consumption/carbon uptake ratio (Videla et al. 2001). These apparently conflicting observations suggest that aging leads to an impairment in some functions of Kupffer cells, as reflected in a substantial reduction in their phagocytosis-stimulated respiratory burst activity, and this may contribute to increased susceptibility of the liver to noxious challenges. (Videla et al. 2001).

Kupffer cell activity is positively involved in the generation of some hepatotoxic effects, thus age-related alteration in Kupffer cell activity may result in a reduced level of injury in response to some toxins. Senescent male Fischer 344 rats have been shown to be resistant to cadmium-induced hepatotoxicity as compared with young adult rats (Yamano et al. 2000). The phagocytic activity of Kupffer cells was stimulated by the administration of cadmium in young adult (5 months) rats but not in old (28 months) rats (Yamano et al. 2000). Observations in that study suggested that (1) Kupffer cell activation is essential for inflammatory liver damage from cadmium, (2) IL-1β and cytokine-induced neutrophil chemoattractant (CINC) are important mediators of the inflammatory response induced by cadmium, and (3) the attenuation of cadmium-induced liver injury in senescent rats is caused by an impairment in Kupffer cell activation, leading to a lower production of CINC and resultant lower level of inflammatory liver injury (Yamano et al. 2000).

5.4.3 Effects of Aging on Dendritic Cells

Lymphoid and myeloid dendritic cells (DCs) are derived from lymphoid and myeloid precursors in the bone marrow, thus are considered to be of hematopoietic origin. The DCs in blood are typically identified by flow cytometry, which reveals three types of DCs in humans: $CD11c^+$ myeloid DCs (mDC), $CD141^+$ myeloid DCs (mDC), and

CD303+ plasmacytoid DCs (pDC). Immature dendritic cells, including the 'veil cells' of circulating blood and lymph, do not have the cytoplasmic 'dendrites' or all the functional characteristics of mature dendritic cells. For laboratory experiments, cultured dendritic cells may be derived from hematopoietic progenitor cells (thus are called HP-DC), or from circulating monocytes (thus are called MODC).

The follicular dendritic cells (FDCs) of secondary lymphoid tissues have "dendritic" cytoplasmic processes that are similar to the processes on hematopoietic DCs, but FDCs are thought to be of mesenchymal rather than hematopoietic origin. Lymphoid and myeloid dendritic cells are 'professional antigen-presenting cells' that present antigenic peptides associated with MHC Class II molecules. By contrast, FDCs do not express MHC Class II. Dendritic processes on FDCs have aggregations of antigens complexed with specific antibodies, the latter attached to the FDC dendrites via FC receptors to form the 'iccosomes' that are visible via electron microscopy, and may be faintly visible by light microscopy. The complexed antigen retained on FDC dendrites serves an important function in prolonging the humoral immune response. The following discussion of age-related effects on dendritic cells is focused on lymphoid and myeloid DCs rather than FDC, except where FDCs are specifically indicated.

Similar to macrophages, dendritic cells play a critical role in linking the innate and adaptive immune systems. They serve as 'professional' antigen-presenting cells in the capture, processing and presentation of antigens in the adaptive immune system, and produce a number of cytokines that modulate the function of other cells of the innate and adaptive immune systems (Weiskopf et al. 2009). Dendritic cells laden with antigenic peptides migrate from areas of inflammation to draining lymph nodes, where the dendritic cells present antigen to T cells and initiate the sequence of events that result in clonal expansion of antigen-specific T cell populations. Aging generally results in decreased dendritic cell (DC) function, though some DC-mediated functions are unaffected by age and some are actually enhanced by age (Shaw et al. 2010).

Data from aged mice suggest there are age-related deficits in the ability of dendritic cells to stimulate the T cell response, probably as a result of reduced dendritic cell expression of chemokine CCR7, which would impair homing to the high-endothelial venules (HEV) of lymph nodes (Grolleau-Julius et al. 2008). Monocyte-origin dendritic cells (MODCs) from aged humans also exhibit impaired migration. Studies have shown pronounced age-related disruption of the follicular dendritic cells (FDC) in primary and secondary follicles, which may be the basis for age-related decline in B cell adaptive responses (Allen and Cyster 2008; Brown et al. 2009; Jing et al. 2009). These alterations in DC may be the basis for the impaired control of viral infections that is observed in aged humans (Shaw et al. 2010).

In addition to age-related deficits in migration, monocyte-origin dendritic cells (MODCs) from aged humans exhibit decreased levels of pinocytosis and phagocytosis in comparison to MODCs from younger subjects (Agrawal et al. 2007). Phagocytosis of apoptotic cells by macrophages and dendritic cells is important in controlling the scope of tissue reactions in areas of inflammation. Thus, in addition to the obvious age-related decrement in ability to assimilate microbial peptides and function as APCs for the adaptive immune system, reduced phagocytic ability may compromise the ability of dendritic cells to remove apoptotic cells. Presence of this apoptotic debris may stimulate an inflammatory reaction, thus its presence contributes

to the pro-inflammatory background seen in aged individuals (Agrawal et al. 2007; Fadok et al. 1998).

Phosphoinositide 3 kinase (PI3K) plays a critical role in both phagocytosis and migration of dendritic cells (Del Prete et al. 2004). PI3K signaling and effector pathways are an important therapeutic target for control of inflammatory and other disease processes. Pharmacologic interruption of PI3K-mediated pathways may have far-reaching effects on overall immunoreactivity and may result in a variety of secondary complications in toxicology studies. (Del Prete et al. 2004; Stephens et al. 2002). An age-related reduction in PI3K activation has been demonstrated in MODCs of aged human subjects (Agrawal et al. 2007).

Aging in rhesus macaques is associated with a shift toward the nonclassical $CD14^{+}CD16^{+}$ monocyte/macrophages phenotype and the $CD11c^{+}$ myeloid dendritic cell population (Asquith et al. 2012).

5.4.4 Effects of Aging on NK Cells

NK cells mediate MHC-independent cytotoxicity against viral infections and some neoplasms. In contrast to T and B cell populations, the absolute number of NK cells increases with age (Miyaji et al. 2000; Borrego et al. 1999). However, the level of NK cell cytotoxicity decreases with age, as do the levels of NK activation-associated cytokines and chemokines such as RANTES, MIP1α, and IL-8 (Mocchegiani et al. 2009). The combination of observations suggest an age-related reduction in the activity of NK cells that is partially offset by an increased number of NK cells in aged individuals. There is evidence that high NK cell activity is associated with healthy aging and longevity, while low NK cell activity is associated with morbidity and mortality due to infections, atherosclerosis, and poor response to immunizations (Solana and Mariani 2000; Solana et al. 1999; Ogata et al. 2001; Bruunsgaard et al. 2001; Molling et al. 2005). These alterations in NK function result, in part, from changes in zinc homeostasis in aged individuals, thus NK function may be improved with zinc supplementation (Mariani et al. 2008; Mocchegiani et al. 2009).

Age-related changes in NK cells of nonhuman primates apparently parallel those seen in humans. An age-related decline in the cytotoxic function of natural killer cells is predictive for impending mortality in aged rhesus macaques (Coe and Ershler 2001).

5.4.5 Effects of Aging on NKT Cells

NKT cells have T cell receptors that are CD1d-restricted, as opposed to the MHC restriction of the T cell receptor on typical T cells, thus represent a type of ‘innate immune lymphocyte’ (Shaw et al. 2010). In aged mice there is an increase in number and function of NKT cells (Faunce et al. 2005; Inui et al. 2002; Kawabata et al. 2008). Increased NKT number and function in aged mice was associated with elevated levels of IL-17A following experimental infection with herpes simplex virus-2

(Stout-Delgado et al. 2009). The older mice also had increased neutrophil recruitment to the liver and increased chemokine production. The enhanced inflammatory environment associated with aging has also been associated with IL-17-dependent T-cell responses (Tesar et al. 2009), thus it appears that IL-17-related aging effects are mediated through both the innate and adaptive immune systems.

5.4.6 Effects of Aging on Mast Cells and Eosinophils

Mast cells are widely distributed in mammalian tissues and upon activation, e.g., by aggregation of FcεRI, produce cytokines and chemokines that influence inflammation and immunologic responses (Galli et al. 2005). Cutaneous mast cells are reduced in number in aged humans, unchanged in aged C57BL/6 mice, and increased in aged BALB/c mice (Hart et al. 1999; Montagna and Carlisle 1990; Nguyen et al. 2005). Jejunal mast cell populations were reported to be unaltered in aged compared to young humans (Arranz et al. 1992), but the mast cell counts in the reported study were performed on toluidine blue-stained sections derived from archived biopsy specimens that may have been preserved by routine fixatives such as formalin. While routine toluidine blue staining on formalin-fixed tissue specimens would reveal classical ('connective tissue') mast cells, it would not reveal mucosal mast cells, which are a distinct subpopulation of mast cells in the mucosa of the gastrointestinal and respiratory tracts (Strobel et al. 1981). Fixation in Carnoy's fixative or basic lead acetate (BLA) preserves the cytoplasmic granules that are necessary for histological identification of mucosal mast cells by histochemical stains and routine light microscopy (Strobel et al. 1981). An immunohistochemistry study of cell populations in the gastrointestinal tract of dogs revealed no age-related change in mast cell subtypes (Kleinschmidt et al. 2008), and immunohistochemical staining would be expected to reveal both connective tissue and mucosal mast cell populations. In summary, it appears that numerical populations of intestinal mast cells are unaffected by aging.

However, mast cells (MCs) are a source of various inflammatory cytokines, chemokines and growth factors, and there is evidence these MC functions are altered by aging. In a study of MC density in liver tissue of 2- and 19-month old rats at 2 and 24 h after carbon tetrachloride (CCl4) treatment, MC population density increased more in the younger rats, suggesting that older rats have a reduced immune response or recruit fewer MCs (Grizzi et al. 2003). Thus the effects of aging on MC are similar to aging effects on other cells of the innate immune system, i.e., little or no effect on cell populations but age-related effects on cell function and responses.

Eosinophils are primarily involved in hypersensitivity reactions and host defense against parasites. They are involved in the pathogenesis of asthma, where the number of eosinophils in airways correlates with disease severity (Trivedi and Lloyd 2007). Eosinophil numbers and functions are affected by aging (Kasper and Haroske 1996; Leng et al. 2005; Mathur et al. 2008; Yagi et al. 1997), suggesting age-related changes in eosinophil function may be involved in the increased severity of asthma that is seen in aged humans (Gomez et al. 2008b; Mathur et al. 2008). Experimental evidence indicates that aged rats fail to accumulate eosinophils in allergic inflammation

of airways (Yagi et al. 1997). As with age-related effects on other cells of the immune system, observations suggest age-related alterations in eosinophil involvement in disease processes is related more to cell function than absolute numbers of cells.

5.5 Effects of Aging on the Adaptive Immune System

Due to the potential for direct impact on immunologically mediated disease processes, there is intense interest in pharmacological intervention in the signaling and effector pathways of the adaptive immune system. Many of these diseases, e.g. rheumatoid arthritis, multiple sclerosis, lymphocytic (Hashimoto's) thyroiditis, type 1 diabetes, and various autoimmune 'collagen diseases', are a direct result of perturbations in immunobiology. In addition to the direct effects on primary immune system functions, immunosenescence also contributes to development or exacerbation of age-related disease such as Alzheimer's disease, atherosclerosis, chronic renal disease, sarcopenia, diabetes, osteoporosis, and rheumatoid arthritis (Ginaldi et al. 2005; Wick et al. 2000; Franceschi et al. 2000; Dietert et al. 2012). Though not necessarily caused by immunobiological dysfunction, therapy of many forms of cancer is augmented by immunotherapy. The breadth and depth of involvement of immunobiology in human diseases is being addressed by the pharmaceutical industry (Henson et al. 2008), to the extent that anyone involved in drug development will benefit from knowledge of immunobiology.

Aging is associated with a multitude of changes in the adaptive immune system, but the major changes can be grouped as (1) lower production of naïve T cells, (2) a shift from effector T cells towards memory T cells, especially memory $CD8^+$ T cells, (3) decrease in $CD4^+/CD8^+$ ratio, (4) decreased diversity of T and B cell populations, and (5) reduction in the number of circulating B cells.

5.5.1 Effects of Aging on T Cells

One of the most direct and immediate effects of aging is the reduction in the population of naïve T cells associated with senescent involution of the thymus. Thymic involution of the thymus, one of the most striking features of immunosenescence, is characterized by a reduction in the overall size of the organ and replacement of cortex and medulla by fat (Weiskopf et al. 2009). Thymic involution in humans starts early in life and is nearly complete by the age of 40–50 years (George and Ritter 1996). Coincident with thymic involution, the number of naïve T cells exiting the thymus is dramatically decreased with age (Fagnoni et al. 2000). However, despite the reduced number of newly formed naïve T cells, a diverse $CD4^+$ (helper) T cell compartment is maintained in humans until approximately age 70, when a dramatic decline in T cell diversity results in a severely reduced $CD4^+$ T cell repertoire (Goronzy and Weyand 2005). A pronounced reduction in diversity of the $CD8^+$ (cytotoxic) T cell population occurs even earlier in humans (Effros et al. 2003). In addition to the numerical deficits in cell

populations and reduced immunological diversity in the T cell population, naïve T cells from elderly persons have numerous structural and functional defects that include significantly shorter telomeres, reduced interleukin-2 production, and impaired ability to expand and differentiate into effector cell populations (Pfister et al. 2006; Kohler et al. 2005; Haynes et al. 2003). A particularly important facet of immunosenescence in humans is decreased T cell proliferative capacity in response to polyclonal stimulation (Miller 2000). This is thought to be due to accumulation of terminally differentiated $CD28^-$ T cells, which have shortened telomeres (Nociari et al. 1999) and impaired ability to induce telomerase (Valenzuela and Effros 2002), resulting in impaired proliferation capability (Effros 2005). Studies have shown similar reduction in T cell proliferative ability in aged rhesus monkeys (Jankovic et al. 2003).

Reduction in the population of naïve T cells is due to several factors. Decreased production of hematopoietic stem cells in the bone marrow leads to decreased migration of early T cell progenitors to the thymus which, coupled with thymic atrophy, leads to a decline in naive T cell production (Chen 2004). In addition, there is an increased rate of conversion of naïve T cells into memory T cells ('homeostatic proliferation') which dramatically increases in humans after 70 years of age (Naylor et al. 2005). Similar shifts in T cell populations are seen in aged rhesus monkeys (Jankovic et al. 2003; Pitcher et al. 2002). In addition to the above factors that favor populations of $CD28^-$ $CD8^+$ T cells, $CD8^+$ T cells in rhesus monkeys undergo more rounds of division than $CD4^+$ T cells (Pitcher et al. 2002; Jankovic et al. 2003), which contributes to the predominance of $CD8^+$ cells that is the basis for alteration in the $CD4^+/CD8^+$ population ratio. Expression of CD28 decreases with the greater number of cell divisions, thus contributing to the selective accumulation of immunologically compromised $CD28^-CD8^+$ T cells in rhesus monkeys (Jankovic et al. 2003).

The age-related reduction in the population of naïve T cells is accompanied by an increase in the number of memory and, particularly, effector T cells (Weiskopf et al. 2009). This increase in memory and effector cell populations, coupled with the reduced number of functionally responsive naïve T cells, has multiple downstream effects on humoral immune responsiveness. Accumulation of $CD8^+$ effector T cells correlates with impaired generation of protective antibody levels in aged humans (Goronzy et al. 2001; Saurwein-Teissl et al. 2002). The accumulating effector T cells have phenotypic changes such as the loss of the co-stimulatory CD28 molecule, which is a key predictor of immune incompetence in aged individuals (Vallejo 2005). In $CD4^+$ T cells, the loss of CD28 is accompanied by reduced CD40L expression, resulting in reduced helper function by $CD4^+$ T cells and associated lower level of B cell proliferation and antibody production (Weiskopf et al. 2009). The increased population of $CD28^-$ T cells produces inflammatory cytokines (Almanzar et al. 2005), thus contributes to 'inflamm-aging' in aged individuals. The $CD28^-$ T cells have increased resistance to apoptosis and restricted T cell diversity, which contribute to the accumulation of long-lived but immunologically compromised T cells (Vallejo 2005; Spaulding et al. 1999). Thymic involution in humans also results in decreased output of regulatory T cells (Tregs) after the age of 50, which may contribute to increased inflammation and autoimmunity (Tsaknaridis et al. 2003).

Activation of B cells and subsequent antibody production involves a sequence of steps involving T_H cells and two co-stimulatory molecules and their ligands: CD40/

CD40L and CD and B7/CD28-CTLA-4. The first step in the induction of the humoral response is binding of antigen to specific immunoglobulin (Ig) on the surface of the B cell. Following internalization of the Ig/Ag complex, fragments of the Ag are presented in association with MHC-II on the surface of the B cell. The antigen complexed with MHC-II is recognized by Ag-specific T cell receptor (TCR) on the T_H cell, with CD4 serving to bind the TCR to the MHC-II molecule on the B cell. The co-stimulatory molecule CD40 is expressed on all B cells, and its ligand (CD40L) is expressed on the T_H cell upon activation by specific antigen in MHC-II context. The CD40-CD40L interaction is necessary for subsequent B cell responses to antigen, and any decrement in CD40 expression would hinder this primary step in the humoral immune response.

Interaction between B7-1/B7-2 (CD80/86) with CD28 or CTLA-4 is the second important step in the T cell/B cell interaction that results in the humoral immune response. The co-stimulatory molecule B7 (CD80/86) is expressed on dendritic cells, activated macrophages and activated B cells, which serve as antigen-presenting cells (APCs). The co-stimulatory molecule CD28, which has moderate affinity for B7, and CTLA-4, which has high affinity for B7, is expressed on T_H cells. The interaction between B7 on APCs and CD28 on T_H cells triggers the release of IL-2, which induces expression of high affinity CLTL-4 by T_H cells. CTLA-4 has 20x the affinity for B7 as does CD28, therefore the association between APCs and T_H cells becomes stronger in this latter stage of activation. The B7/CD28 interaction has a positive effect on the humoral immune response, while B7/CTLA-4 interaction has a damping effect on the humoral response and helps to control auto-reactivity (Rudd and Schneider 2003; Rudd et al. 2009; Chikuma and Bluestone 2003; Greenwald et al. 2005). Presence of a population of T cells with deficient CD28 expression would significantly reduce the initial interaction between antigen-presenting cells and T_H cells, thus would intercept the downstream events that promote humoral immunity.

In addition to promoting humoral immune responses, B7-1/B7-2:CD28 interactions also regulate self-tolerance by maintaining homeostasis of $CD4^+CD25^+$ Treg cells (Greenwald et al. 2005). Additional members of the B-7 family such as inducible costimulatory molecule (ICOS) ligand, programmed cell death ligand 1 (PD-L1) (also known as B7-H1), PD-L2 (also known as B7-DC), B7-H3, and B7-H4 (also known as B7x & B7-S1) are expressed on APCs and nonlymphoid cells, thus providing additional avenues for regulation of T cell activation and tolerance in peripheral tissue (Greenwald et al. 2005). Additional members of the CD28 family, ICOS, PD-1, and BTLA (B and T lymphocyte attenuator) are inducibly expressed on T cells, and join with CTLA-4 signaling in regulating previously activated T cells (Greenwald et al. 2005). PD-1 and BTLA are also expressed on B cells, and the PD-1: PD-L1/PD-L2 pathways have a critical role in regulating T cell activation and tolerance (Greenwald et al. 2005). There is an increase in the ratio of inhibitory T follicular regulatory (T_{FR}) cells to stimulatory T follicular helper (T_{FH}) cells in aged mice, which exhibit increased programmed cell death protein-1 (PD-1) expression but decreased ICOS expression (Sage et al. 2015). As compared to younger mice, aged mice have an increased number of $CD4^+$ T cells that express the down-regulatory PD-1, ICOS, and CTLA-4 molecules (Channappanavar et al. 2009).

Both the B-7/CD28 and CD40/CD40L co-stimulatory pathways have been shown to become dysfunctional with aging (Weiskopf et al. 2009; Effros 2000). Aged humans and mice have an increased number of memory T cells that exhibit a marked reduction in responsiveness to antigen stimulation (Nagelkerken et al. 1991; Utsuyama et al. 1992; Ernst et al. 1990; Lerner et al. 1989). Much of the hyporesponsiveness is attributable to changes in the interaction between CD80/86 and CD28 (Engwerda et al. 1994). In addition to effects mediated via the CD80/86 and CD28 interactions, naïve T cells of aged subjects have a generalized reduction in responsiveness that is antigen-independent, suggesting autonomous age-related changes in the T cell population (Linton et al. 1996; Linton and Dorshkind 2004).

In addition to effects on the $CD4^+$ T_H cell population, there are also age-related effects on the $CD8^+$ T_C cell population. With aging there is an increase in the population of $CD28^-$ T cells which, in conjunction with the higher turnover rate in the $CD8^+$ T cell subset relative to the $CD4^+$ subset, results in an increased relative population of $CD28^-$ $CD8^+$ Tc cells (Sansoni et al. 2008; Vallejo 2005).

Prolonged immunologic stimulation by pathogens, particularly viruses, plays a substantial role in modulating immune responsiveness in humans, and would be expected to have similar effects in animal species. Among the persistent viral pathogens that impact on the human immune system, the herpesvirus family (cytomegalovirus, Epstein-Barr virus, and herpes simplex virus) is most prominently involved in modulation of T cell populations in aged subjects (Wikby et al. 2005; Koch et al. 2007)(ref41-(Nikolich-Zugich 2008). Cytomegalovirus is a herpesvirus that establishes lifelong persistence in 60–100% of the human population ((Zinkernagel 1996; Looney et al. 1999). Cytomegalovirus-associated clinical disease can occur in immunosuppressed patients, but immunocompetent persons typically do not experience clinical disease even though the immune system declines with age (Weiskopf et al. 2009). However, there is evidence that lifelong viral persistence in the immunocompetent host may accelerate aging of the immune system and may lead to chronic subclinical inflammation (Almanzar et al. 2005). Up to 25% of the total $CD8^+$ T cell pool of aged individuals may be specific for cytomegalovirus, thus reducing the availability of T cells that are capable of responding to other pathogens (Gillespie et al. 2000; Khan et al. 2002). These processes contribute to the decline in $CD4^+/CD8^+$ ratio, which is a hallmark feature of immunosenescence in humans (Moro-Garcia et al. 2013; Channappanavar et al. 2009). There is evidence that cytomegalovirus infection persists in susceptible nonhuman primate colonies. In a serological study of rhesus monkeys in a colony that had been closed to new animal introductions for 70 years, 95% of specimens were serologically positive for cytomegalovirus (Andrade et al. 2003). Similar to the situation in humans, studies in rhesus monkeys also revealed a progressive decline in $CD4^+$:$CD8^+$ ratio between 2 months and 5 years of age, with no further decline between 5 and 7 years of age (Dykhuizen et al. 2000) or at 7–15 or 18–30 years of age (Haberthur et al. 2010).

In addition to the generation of T cells, the thymus is the source of multiple hormones that are involved in post-thymic T cell maturation (Aita et al. 1981; Savino et al. 1983b, a). Circulating levels of thymic hormones reach an apex soon after birth (Dardenne et al. 1988; Naylor et al. 1984), after which there is a gradual decline in the circulating level of thymic hormones (Naylor et al. 1984) and a

decrease in the number of thymic epithelial cells that secrete thymic hormones (Hirokawa et al. 1982; Schuurman et al. 1985).

Age-related changes in T cell populations of nonhuman primates are similar to those seen in humans. The most pronounced age-related changes in nonhuman primates are reduced numbers of naïve T cells, declining $CD4^+$: $CD8^+$ T cell ratios, and increased number of terminally differentiated memory T cells, many of which are specific for persistent viral infections (Cicin-Sain et al. 2007; Didier et al. 2012; Jankovic et al. 2003).

5.5.2 Effects of Aging on B Cells

Activation of B cells includes a sequence of processes: (1) B cell presentation of processed antigen along with class II MHC molecule to activated T_H cells specific for that antigen, (2) formation of a T_H/B cell conjugate, which induces expression of CD40L on the T_H cell membrane and directional release of cytokines by the T_H cell to the B cell, and (3) generation of signals in B cells that lead to proliferation and differentiation (Goldsby et al. 2000). Aging is associated with a decline in B cell function and impairment of the antibody response. While much of this decline in B cell function is attributable to declining T_H cell helper function, the B cell compartment undergoes age-related changes that are independent of the T cell compartment (Ademokun et al. 2010; Cancro et al. 2009; Frasca and Blomberg 2009). The number of B cells declines with age due to a decline in B cell precursors (Frasca and Blomberg 2009), and there is evidence that aging results in the accumulation of memory B cells at the expense of naïve B cells (Colonna-Romano et al. 2008). The percentage of naïve B cells, as defined by the absence of CD27, is significantly reduced in aged humans (Weiskopf et al. 2009).

In addition to reduction in the number of B cells, aging is also associated with multiple changes in the function of the remaining B cell population. The memory B cells that accumulate in aged humans have decreased susceptibility to apoptosis (Chong et al. 2005), leading to clonal expansions of certain B cell specificities (Weksler 2000; Weksler and Szabo 2000), which serves to limit the diversity of the B cell repertoire. The decrease in B cell repertoire diversity correlates with clinical frailty in humans (Gibson et al. 2009). Antibodies generated in old age are also of lower affinity due to a shift in antibody isotypes from IgG to IgM (Johnson and Cambier 2004).

B cells depend on interaction with other cells for full functionality, therefore, age-related alterations in B cell function are likely to result from a combination of effects on B cells and dysregulation of the function of other cell types within the immune system. For example, B cells from elderly individuals are stimulated less efficiently by follicular dendritic cells than B cells from young subjects (Aydar et al. 2002) as a result of the decreased expression of co-stimulatory molecules such as CD40 or CD27 (Colonna-Romano et al. 2003). The $CD4^+$ T cells of aged individuals produce less IL-2 and express less CD40L, which is crucial to the interaction between T cells and B cells (Weiskopf et al. 2009). Less efficient T cell help and the altered cytokine environment result in defects in antibody production (Miller et al.

1995), and is likely to lead to reduced B cell expansion and differentiation in response to antigens (Lazuardi et al. 2005).

Effects of aging on long-lived animal species are more difficult to determine than changes in rodents, where 2 years constitutes a lifetime, but sparse available information suggests aging is associated with B cell decrements in nonhuman primates that are similar to those observed in humans. Aged rhesus monkeys (>18 years old) have fewer circulating B cells than young adults (5–10 years old), and the frequency of antigen-experienced CD27+ (memory and marginal zone-like) B cells increases with age (Haberthur et al. 2010). The repertoire of circulating B cells also declines with aging of rhesus macaques (Didier et al. 2012; Haberthur et al. 2010).

5.5.3 Effects of Aging on the Germinal Center Reaction

Studies in mice have revealed that aged animals do not exhibit the increase in affinity of primary antibody response that is seen in young animals. These reductions in humoral immune responses correlate with 60–95% reductions in germinal center reactions in aged mice (Szakal et al. 1990; Kosco et al. 1989). The reduction in germinal center reactions in mice is a gradual process that is discernible as early as 6 months of age, and becomes progressively more pronounced with increased age (Zheng et al. 1997).

Diminished GC response with aging is partially due to defective transport of antigens to lymphoid follicles and subsequent functional impairment of the antigen-responsive reticulum (ARR). Studies in mice have shown an age-related reduction in the number of antigen transport sites and diminished ARR (Szakal et al. 1990; Szakal et al. 1988). Additionally, most of the antigen transport sites in aged mice had defective antigen transport cells, and the ARR contained atrophic FDCs that could not produce iccosomes effectively (Szakal et al. 1990). These factors may explain observations of reduced GC reactions in aged versus young mice following antigenic challenge (Szakal et al. 1988).

Signaling through the CD40-CD40L pathway appears to direct germinal center B cells toward the memory B cell pathway, while the absence of CD40-CD40L signaling results in germinal center centrocytes becoming terminally differentiated, immunoglobulin-producing plasma cells (Arpin et al. 1995). The opposing signaling pathway that is mediated via OX40 on activated T_H cells and OX40L on activated B cells directs activated B cells to become plasma cells (Stuber et al. 1995; Stuber and Strober 1996). Therefore, relative predominance of the OX40-OX40L pathway, which would exist by default with an aging-related decrease in the CD40-CD40L pathway, would favor short-lived antibody production responses rather than long-lived memory responses.

Antibody production in the bone marrow occurs late in the primary response, but is much more important during secondary immune responses (Benner et al. 1974). Germinal center B cells migrate from secondary lymphoid tissues to the bone marrow, where they produce the large amount of specific antibody that is typically associated with secondary immune responses (Benner et al. 1974; Koch et al. 1981; Smith et al. 1997). Presence of long-lived antibody-forming cells in

the bone marrow is dependent on the germinal center reaction (Han et al. 1995a; Foy et al. 1993; Han et al. 1995b) which, in turn, is dependent on generation of high-affinity antibody-forming cells by intact CD40-CD40L signaling (Takahashi et al. 1998, press). With deficient CD40-CD40L signaling, the germinal center reaction does not produce the high-affinity antibody-forming cell population that secondarily populates the bone marrow with long-lived antibody-producing cells, thus resulting in the short-term immunologic responses seen in the elderly.

Generation of a protective immune response typically involves a process of affinity maturation within germinal centers, which is a result of antigen-driven somatic hypermutation of immunoglobulin genes and selection of B cells with high affinity antigen receptors (Jacob et al. 1991; Berek et al. 1991). Functional input from $CD4^+$ T_H cells is necessary for both germinal center formation and activation of the immunoglobulin somatic hypermutation machinery (Miller et al. 1995; Maizels and Bothwell 1985). Senescence of T_H cell populations appears to play a pivotal role in age-related alterations in somatic hypermutation of immunoglobulin genes (Zheng et al. 1997).

5.6 Effects of Aging on Cytokines

Some observations suggest aging in humans is associated with an increase in pro-inflammatory plasma cytokine levels, specifically IL-6 and TNF-α (Franceschi et al. 2000), while other studies have shown no such effect (Kaack et al. 1998; Hasegawa et al. 2000). The differences in results may have been due to the health of the subjects (Haberthur et al. 2010). While elevated IL-6 levels correlate with several chronic diseases of humans (Bruunsgaard 2002; Ershler and Keller 2000; Huang et al. 2005), it is not clear whether the increased cytokine level is a cause or a result of the inflammatory diseases. One study revealed that aged female rhesus monkeys had higher plasma levels of TNF-α and IL-8, but no differences in IL-1α, IL-2, or IL-6 levels (Kaack et al. 1998). It has been postulated that removal of clinically frail animals due to animal welfare considerations results in an unusually healthy population of geriatric monkeys that may not be equivalent to the human population (Haberthur et al. 2010). In contrast to the previous observations, it has been shown that release of IL-6 by endothelial cells, either spontaneously or in response to inflammatory cytokines and hypoxia, increases with age (Coe 2004).

Rhesus macaques, as humans, have age-related increases in circulating TNF-α, IL-6, and IFN-γ (Didier et al. 2016).

5.7 Clinical Effects of Immunosenescence

As a result of the combined decrements in various components of the innate and adaptive immune systems, there is a general decline in immune responses with aging. Primary antibody responses in aged humans tend to be weaker and shorter-lived than those in young subjects (Miller 1991; Weksler and Hutteroth 1974;

Kishimoto et al. 1980). After immunization, aged individuals tend to generate smaller quantities of antibodies (Miller 1991; Weksler and Hutteroth 1974; Burns et al. 1990), maintain protective antibody titers for a shorter period of time (Kishimoto et al. 1980), and produce antibodies with lower affinity and/or avidity than those produced by young adults (Goidl et al. 1976; Kishimoto et al. 1976; Doria et al. 1978; Weksler et al. 1978). Therefore, in aged animals and elderly humans the quality of antibodies produced may be insufficient for disease protection even though the initial response to a specific antigen is robust (Burns et al. 1993; Nicoletti et al. 1991; Nicoletti et al. 1993).

Lower level of mucosal immunity in aged nonhuman primates results in lower IgA responses to cholera toxin/toxoid (Taylor et al. 1992), possibly due to age-related alteration in the expression of homing molecules that result in reduced emigration of IgA immunoblasts from Peyer's patches to the small intestinal lamina propria (Schmucker et al. 2003).

Spontaneous age-related decline in immune system function may be exacerbated by other factors. Psychosocial stress associated with lower social status in aging rhesus macaques involves a link between the hypothalamic-pituitary-adrenal axis, immune senescence and reproductive stress, and results in higher levels of cortisol, increased proinflammatory cytokine levels and lower natural killer cytotoxic activity in psychosocially stressed animals (Coe 2004; Coe and Ershler 2001; Coe et al. 1992; Goncharova and Lapin 2004; Hoffman et al. 2011; Hoffman et al. 2010). These negative effects of the social environment can be counteracted by positive social influences, as evidenced by reduction in the effects of psychosocial stress in rhesus and Japanese macaques living in colonies that promoted intergenerational relationships and support groups among females (McDonald Pavelka 1994).

5.8 Histomorphology of Immunosenescence

The pathology evaluation of nonclinical toxicology studies typically involves gross necropsy examination, organ weight analysis, clinical pathology tests (hematology, coagulation, clinical chemistry, and urinalysis), and histopathological evaluation of immune system organs. The latter typically includes microscopic examination of thymus, spleen, multiple lymph nodes, and histologic sections of bones containing bone marrow (commonly the sternum and distal femur with femorotibial articulation). Histopathological evaluation of gut-associated lymphoid tissue (GALT) is often required by study protocols. Depending on the species of animal involved, bronchus-associated lymphoid tissue (BALT) may be present in histologic sections of lung and nasopharynx-associated lymphoid tissue (NALT) may be present in histologic sections of nasal cavity. Smears of bone marrow are commonly prepared at necropsy, and cytological evaluation of the bone marrow smears is performed when indicated. Readily discernible histologic alterations associated with aging are commonly present in the thymus, bone marrow, lymph nodes, and spleen. Less pronounced age-associated histologic changes are present in the various components of mucosa-associated lymphoid tissue (MALT).

5.8.1 *Histomorphological Effects of Aging on the Bone Marrow*

Reduction in marrow cellularity is commonly observed in aged rodents, and is typically accompanied by an increase in the population of marrow cavity adipocytes (Fig. 5.1a, b). The decrement in marrow populations is typically divided between erythroid and myeloid elements unless chronic inflammation in peripheral tissues promotes expansion of the myeloid population in the bone marrow. The earliest loss of hematopoietic cells from the sternum is typically seen in the central aspect of the sternebral cavity, with relative sparing of cell populations near the ends of the cavity. Loss of hematopoietic cells from the femur is typically most pronounced in the diaphyseal region of the femur, with less pronounced reduction in hematopoietic activity in the metaphyseal region.

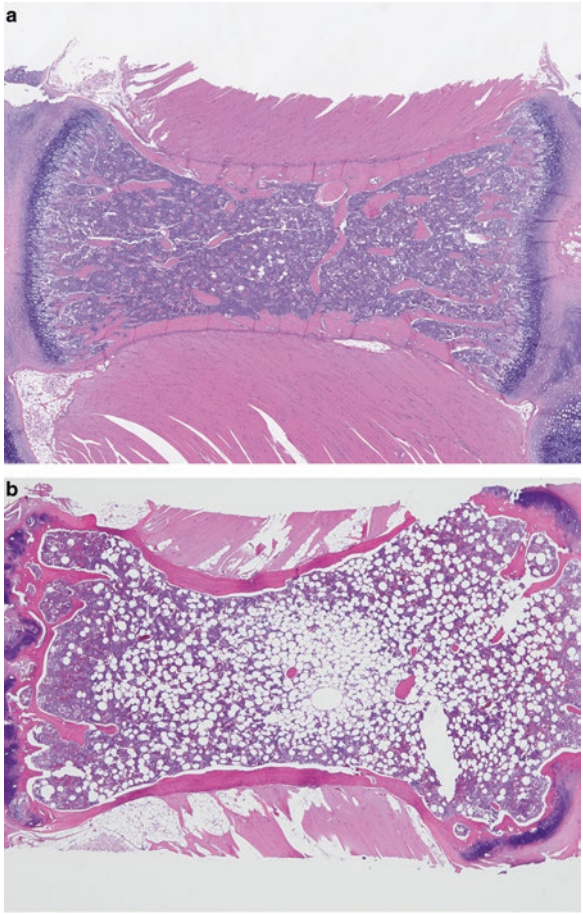

Fig. 5.1 (**a**) The sternal bone marrow of a young (postnatal day 42) male Sprague Dawley rat has uniformly high cellularity throughout the marrow cavity. Hematoxylin & eosin stain, 2.5× objective magnification. (**b**) The sternal bone marrow of an aged untreated control Wistar-HAN rat collected at termination of a 30-month study has a large number of adipocytes with clear cytoplasm within the marrow cavity. Note the typical concentration of adipocytes in the central region of the sternebral marrow cavity. Hematoxylin & eosin stain, 4× objective magnification

5.8.2 *Histomorphological Effects of Aging on the Thymus*

Age-associated thymic involution is one of the most dramatic and ubiquitous changes in the immune system (Aw et al. 2008), and precedes reduced functioning of the immune system. Age-associated thymic atrophy results in a decline in the output of naïve T cells, and has been identified as one of the key events of declining immune system functioning in later life. Studies in C57BL/10 mice show an 83% reduction in the number of T cells between 3 and 20 months of age, with a significant decline in both CD4 and CD8 subsets (Aspinall 1997). The similar percentage decrement in each subset at 3, 12, and 20 months of age suggests a disruption in the early T cell developmental pathway. Reduced T cell production by the thymus apparently results from a failure of the thymic epithelial cell population to support thymopoiesis in old age, and experimental evidence suggests that a decline in interleukin-7 (IL-7) expression may be the key factor (Andrew and Aspinall 2002). The decrement in IL-7 production is not accompanied by a concurrent decrement in Connexin 43 expression, which is a generic marker used to identify thymic epithelial cells, therefore, it appears the reduction in IL-7 production is not due to a numerical reduction in the population of thymic epithelial cells (Andrew and Aspinall 2002). The final effector mechanism that results in altered populations of T cells is reported to be mediated by the death receptor Fas (Yajima et al. 2004).

While it is well established that the thymus is an essential organ for the support of T-cell differentiation, extrathymic T cells have been found to differentiate without thymic support (Abo 2001). The major sites of extrathymic T cell development are the intestine and liver, but extrathymic T cells are also present in the uterus and exocrine glands (e.g., the salivary gland). Extrathymic T cells have a TCR-CD3 complex that is similar to thymus-derived T cells, and the TCR complex has the same αβ and γδ subunits that are seen in thymus-derived T cells. However, extrathymic T cells have some distinctive properties (Abo 1993), including a larger proportion of γδ T cells, presence of double-negative ($CD4^-CD8^-$) cells and self-reactive oligoclones, constitutive expression of the IL-2 receptor beta chain, and common presence of an αα homodimer of CD8 (Abo 1993). Although there are few extrathymic T cells in young animals, this cell population increases in number with age, apparently in parallel with thymic involution. Extrathymic T cells are also increased in number and function by stress, in autoimmune diseases, and during pregnancy. Acute thymic atrophy typically accompanies this physiologically driven extrathymic T cell response, suggesting a reciprocal regulation between extrathymic T cells and thymus-derived T cells.

In a comparison of levels of cellular proliferation and apoptosis in the thymus of 3- and 18-month-old male Wistar rats, in the 18-month-old rats the volume of lymphoepithelial thymic tissue was markedly reduced, reflecting a sharp decrease in the total number of thymocytes (Plecas-Solarovic et al. 2006). A reduction in the proliferative capacity of thymocytes and increase in the rate of apoptosis were considered to be primarily responsible for the observed 7-fold reduction in thymic cellularity in old animals (Plecas-Solarovic et al. 2006). Only the cortical volume was reduced,

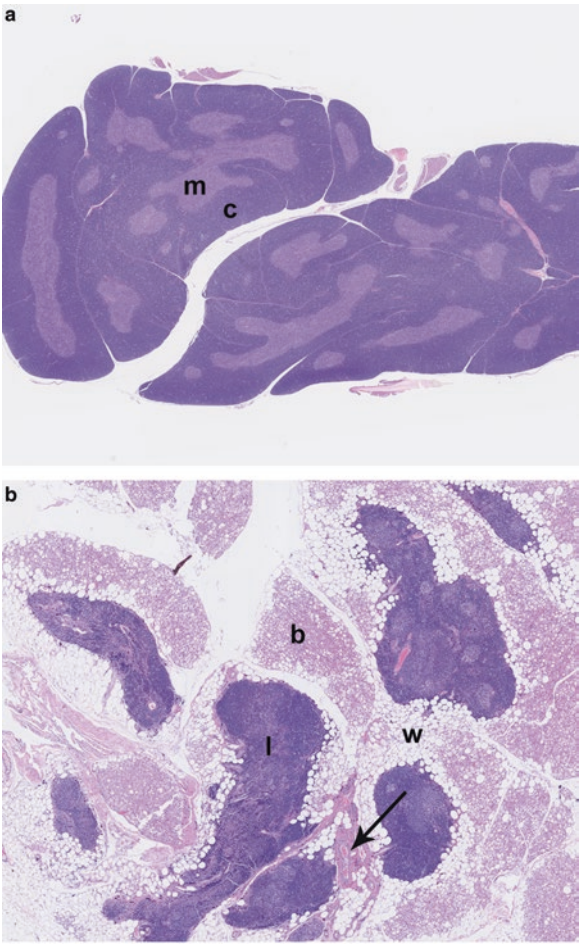

Fig. 5.2 (**a**) The thymus of a young (postnatal day 42) female Sprague Dawley rat has highly cellular cortex (C) and less cellular medulla (M), with a sharply defined boundary between the two regions. Hematoxylin & eosin stain, 1.25× objective magnification. (**b**) The thymus of an aged untreated control male Wistar-HAN rat collected at termination of a 30-month study consists of atrophic lymphoid lobules (l) surrounded by fat with a small amount of fibrous connective tissue and occasional blood vessels (*arrow*). The fatty tissue has a mixture of *white* (w) and *brown* (b) fat. The lymphoid lobules have lightly stained areas that represent medullary tissue, but the organ lacks the orderly corticomedullary architecture that is seen in younger rats. Hematoxylin & eosin stain, 2.5× objective magnification

while the volume of the medulla remained relatively constant despite lower cellularity of the medulla (Plecas-Solarovic et al. 2006).

Pronounced age-associated histomorphologic changes are present in the thymus of rats (Burek 1978; Kuper et al. 1992). Commencing near the time of puberty, though not necessarily associated with puberty, there is a gradual reduction in cellularity of the thymic cortex and less striking reduction in cellularity of the thymic medulla (Fig. 5.2a, b). The reduction in cortical cellularity is more pronounced near septa than near the capsule (Sainte-Marie and Peng 1986). The histologic severity of thymic involution is strain-related, and tends to be more pronounced in males (Burek 1978; Stefanski et al. 1990). Aging changes of the thymus are most commonly observed in rodents from 2-year carcinogenicity studies, where the thymus has a marked decline in cellularity as compared to young animals. However, age-associated histomorphologic alterations may be encountered in the thymus of rats from studies of shorter duration. Assuming that rats are acquired for nonclinical toxicology studies at 6–7 weeks of age and acclimated to laboratory conditions for

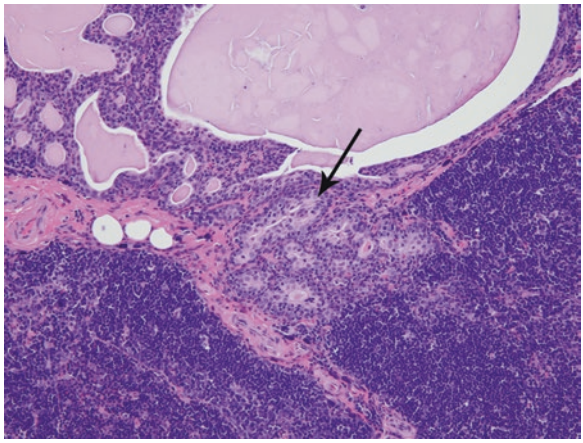

Fig. 5.3 The thymus of an aged female Sprague Dawley rat has epithelial nests and tubules (*arrow*) contained within the medullary region. Hematoxylin & eosin stain, 20× objective magnification

1–2 weeks before experimental manipulation, it is possible for the animals to exhibit some evidence of age-related thymic involution as early as the terminal necropsy of 90-day toxicology studies (rats of 140–155 days of age). Age-related thymic changes are expected at termination of 6-month toxicity studies, are well established at the end of 1-year toxicity studies, and typically are extensive at termination of 2-year carcinogenicity studies.

In addition to reduction in cellularity, the thymus of aged rats of some strains also has clusters of epithelial cells that commonly form nests and tubules (Fig. 5.3), the latter sometimes forming cystic cavities filled with proteinic material (Fig. 5.4). There are strain-related differences in the incidence and prominence of the epithelial nests and tubules, which are more abundant in females (Burek 1978). Ultrastructural study reveals the epithelial structures have a basement membrane and sometimes contain homogeneous eosinophilic secretory material that has no identifying ultrastructural features (Burek 1978). The epithelial cells have large, membrane-bound mucous-like cytoplasmic granules, a second population of smaller, electron-dense cytoplasmic granules, and intercellular desmosomes that connect adjacent epithelial cells (Burek 1978). The epithelial nests and tubules are not present in the thymus of young rats, thus the appearance of the structures in the thymus of aged rats apparently results from proliferation rather than a simple increase in visibility due to involution of the lymphoid component of the thymus. Given the histologic appearance of the structures and the known involvement of the neural crest in formation of the thymic medulla (Bockman and Kirby 1985; Jones et al. 1998), some relationship between the thymic nests/tubules and the neural crest or its derivatives seems likely.

The thymus of aged rats commonly has a modest population of mast cells, which are most commonly present near the capsular surface (Fig. 5.5). Somewhat surprisingly, the thymus of aged rats may also contain plasma cells (Fig. 5.6).

Age-associated thymic involution has the expected effect on organ weight. In a study of age-related changes in the spleen and thymus of male Fischer F344 rat, a decline in thymus weight was most dramatic between 4 and 20 months of age (Cheung and Nadakavukaren 1983). In that study, the cellularity (cells/gram of tissue)

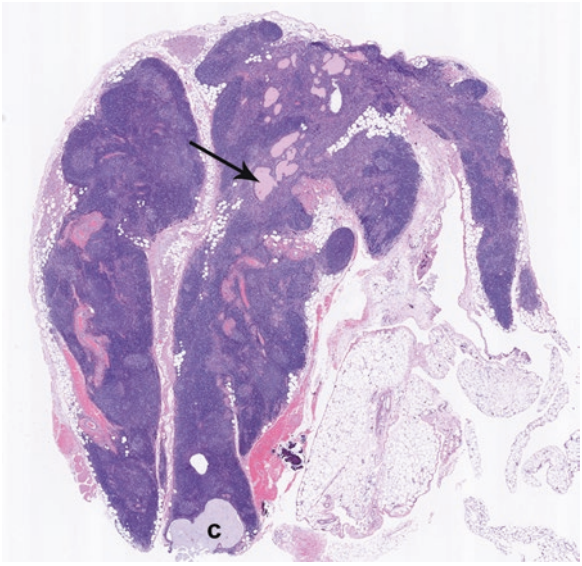

Fig. 5.4 The thymus of an aged untreated control Wistar-HAN rat collected at termination of a 30-month study has thin cortical regions, but retains the overall corticomedullary microarchitecture. Multiple spaces filled with lightly eosinophilic material (*arrow*) represent dilated tubules that commonly develop in the medullary region of aged rats. A larger cystic cavity (c) filled with amphophilic material is a dilated remnant of the embryonic duct system. Note the extensive areas of fatty tissue associated with the thymus. Hematoxylin & eosin stain, 1.25× objective magnification

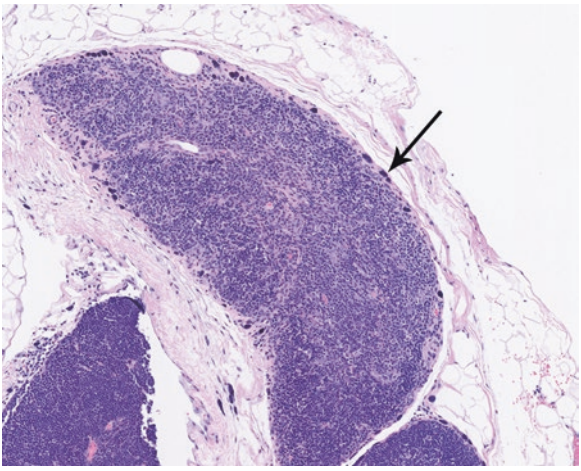

Fig. 5.5 One major lobe of the thymus of an aged untreated control Wistar-HAN rat collected at termination of a 30-month study has a generalized reduction in cellularity, with little definition between cortical and medullary regions. Note the large deeply basophilic mast cells (*arrow*) located beneath the capsule of the thymus. Hematoxylin & eosin stain, 10× objective magnification

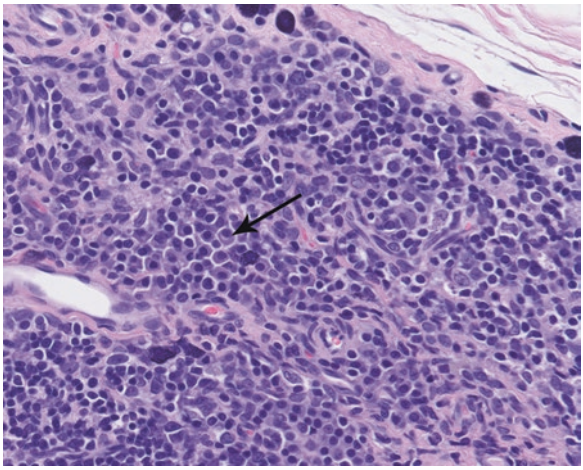

Fig. 5.6 Even though the thymus is the primary organ for T cell maturation, it sometimes contains B cells and plasma cells. A higher magnification of the thymic lobule shown in Fig. 5.4 reveals a moderate number of plasma cells (*arrow*) that are characterized by eccentrically placed nuclei and lightly stained paranuclear zones that represent Golgi zones. Hematoxylin & eosin stain, 40× objective magnification

of both the thymus and spleen declined with age. Table 5.1 presents thymus weights of female and male Crl:CD (SD) rats at various ages as reflected in our historical control data (WIL Research Historical Control Database, Version 3.6, 07Jul2014). The data show 81 and 88% decline in absolute thymus weight of female and male Sprague-Dawley rats, respectively, between the earliest weights (7–8 weeks) versus weights collected at the terminal necropsy of carcinogenicity studies (age = 101–110 weeks). The apparent slight preservation of thymic weight in aged female Sprague-Dawley rats may be complicated by the higher incidence of thymic epithelial tubules and nests in females. There is strain-related variation in the age-related decrement in thymus weight. In one reported study the loss of functional tissue in the thymus of aged rats was compensated by a substantial increase in the volume of connective and adipose tissue, with the result that the thymic weight remained unaltered in aged male Wistar rats (Plecas-Solarovic et al. 2006). Thymic weight data from aged rats must be viewed with some circumspection, as histologic examination of the organs commonly reveals the thymic specimen to consist of a mixture of lymphoid elements and fat (*see* Fig. 5.2b).

Age-associated thymic changes are less pronounced in dogs and non-human primates from standard toxicology studies because the animals typically are not observed at advanced age.

Table 5.1 Thymus weights, Sprague-Dawley rats

		Females		Males	
Age of rats	Organ weights	Mean	SD	Mean	SD
7–8 weeks	Absolute OW (g)	0.4957	0.09840	0.6088	0.13291
	OW relative to FBW (g/100g)	0.291	0.0555	0.258	0.0564
	OW relative to brain	28.131	5.2276	33.050	6.8382
9–12 weeks	Absolute OW (g):	0.4561	0.10825	0.5147	0.13476
	OW relative to FBW (g/100g)	0.209	0.0507	0.153	0.0447
	OW relative to brain	24.673	5.9006	26.136	6.7888
13–15 weeks	Absolute OW (g)	0.3876	0.09712	0.4287	0.11844
	OW relative to FBW (g/100g)	0.156	0.0393	0.102	0.0294
	OW relative to brain	20.416	5.1272	21.044	5.7807
16–18 weeks	Absolute OW (g)	0.3159	0.08563	0.3508	0.08716
	OW relative to FBW (g/100g)	0.118	0.0302	0.073	0.0178
	OW relative to brain	16.389	4.2754	16.629	3.9690
19–21 weeks	Absolute OW (g)	0.2573	0.06402	0.3077	0.08379
	OW relative to FBW (g/100g)	0.095	0.0236	0.060	0.0164
	OW relative to brain	13.242	3.2550	14.485	3.9254
22–25 weeks	Absolute OW (g)	0.2217	0.05725	0.2565	0.07521
	OW relative to FBW (g/100g)	0.076	0.0195	0.046	0.0131
	OW relative to brain	11.360	2.9585	12.027	3.5150
32–34 weeks	Absolute OW (g)	0.1538	0.04824	0.1533	0.04793
	OW relative to FBW (g/100g)	0.050	0.0142	0.026	0.0083
	OW relative to brain	7.846	2.3880	7.031	2.1596
2-yr study	Absolute OW (g)	0.0962	0.04569	0.0710	0.03588
	OW relative to FBW (g/100g)	0.023	0.0085	0.012	0.0060
	OW relative to brain	4.817	2.3079	3.223	1.6406

FBW final body weight; *OW* organ weight; *SD* standard deviation

5.8.3 Histomorphological Effects of Aging on the Spleen

In a study of age-related changes in the thymus and spleen of male F344 rats, the spleen weight was found to increase with age (Cheung and Nadakavukaren 1983). The increase in spleen weight was almost linear between 4 and 30 months of age, yet the lymphoid cellularity (cells/gram of tissue) of the spleen decreased with age. The white pulp of 4-month-old male F344 rats contained a large number of small lymphocytes, and the number of lymphocytes was found to decrease with increasing age. Despite the increase in spleen weight, at 30 months of age the white pulp lymphocyte population was 80% lower than that seen in 4-month-old rats, and the white pulp exhibited an increased number of reticular cells and macrophages with enlarged cytoplasm. This apparent dissociation between spleen weight and spleen cellularity may be explained by the development of mononuclear cell leukemia, which is a very common spontaneous disease process in F344 rats and typically involves the spleen in advance of other organs (Stefanski et al. 1990).

Table 5.2 Spleen weights, Sprague-Dawley rats

		FEMALES		MALES	
Age of rats	Organ weights	Mean	SD	Mean	SD
5–8 weeks	Absolute OW (g)	0.43	0.079	0.60	0.122
	OW relative to FBW (g/100g)	0.257	0.0368	0.260	0.0400
	OW relative to brain weight (g/100g)	24.541	4.2492	32.419	6.3005
9–12 weeks	Absolute OW (g)	0.50	0.089	0.69	0.123
	OW relative to FBW (g/100g)	0.229	0.0326	0.202	0.0321
	OW relative to brain weight (g/100g)	26.902	4.4618	34.833	5.6714
13–15 weeks	Absolute OW (g)	0.54	0.096	0.77	0.126
	OW relative to FBW (g/100g)	0.217	0.0339	0.184	0.0263
	OW relative to brain weight (g/100g)	28.249	4.8885	37.660	5.8717
16–18 weeks	Absolute OW (g)	0.53	0.079	0.82	0.130
	OW relative to FBW (g/100g)	0.199	0.0262	0.171	0.0230
	OW relative to brain weight (g/100g)	27.648	3.8803	38.830	5.9278
19–21 weeks	Absolute OW (g)	0.52	0.087	0.83	0.140
	OW relative to FBW (g/100g)	0.191	0.0277	0.162	0.0222
	OW relative to brain weight (g/100g)	26.764	4.2750	38.956	6.3069
22–25 weeks	Absolute OW (g)	0.53	0.081	0.85	0.127
	OW relative to FBW (g/100g)	0.184	0.0239	0.153	0.0191
	OW relative to brain weight (g/100g)	27.367	4.0599	39.840	5.8748
32–34 weeks	Absolute OW (g)	0.54	0.083	0.86	0.151
	OW relative to FBW (g/100g)	0.174	0.0241	0.144	0.0198
	OW relative to brain weight (g/100g)	27.286	4.0764	39.645	6.7767
2-yr study	Absolute OW (g)	0.82	0.362	1.16	0.376
	OW relative to FBW (g/100g)	0.219	0.0938	0.192	0.0611
	OW relative to brain weight (g/100g)	41.069	18.5301	52.924	17.4462

FBW final body weight; *OW* organ weight; *SD* standard deviation

Table 5.2 presents spleen weights of Crl:CD (SD) rats at various ages as reflected in our historical control data (WIL Research Historical Control Database, Version 3.6, 07Jul2014). In contrast to thymus weights, there is no age-related decrement in spleen weights of males or females. Increased spleen weights in the aged animals at the end of 2-year studies is associated with a variety of pathologic processes that involve the spleen. Note the marked increase in standard deviation in spleen weight parameters of animals at the 2-year interval, which suggests major variations in organ weights in individual animals.

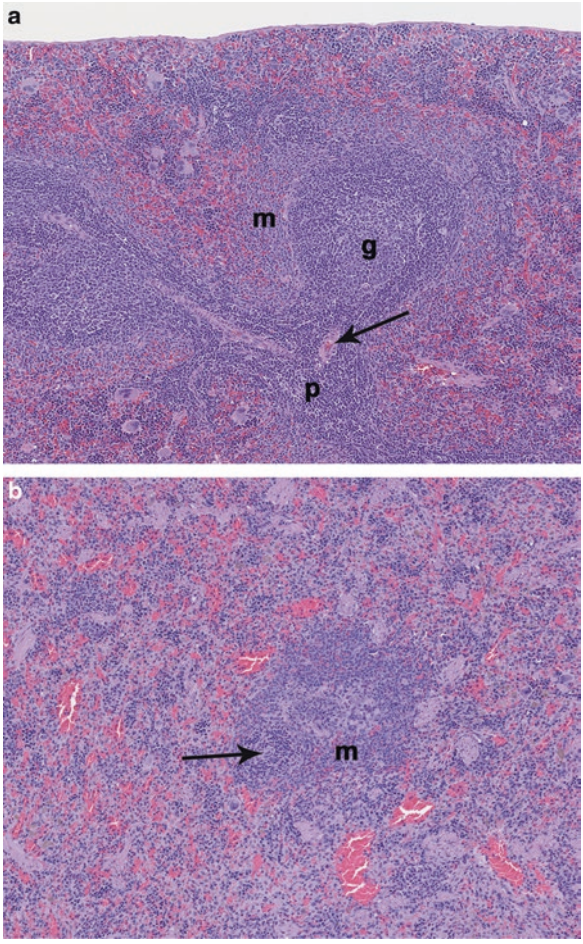

Fig. 5.7 (**a**) The spleen of a young (postnatal day 42) female Sprague Dawley rat has an arteriole (*arrow*) surrounded by a periarteriolar lymphoid sheath (PALS) (p). Secondary follicles containing prominent germinal centers (g) indicate an active humoral immune response. M = marginal zone. Hematoxylin & eosin stain, 10× objective magnification. (**b**) The spleen of an aged untreated control Wistar-HAN rat collected at termination of a 30-month study has pronounced lymphoid atrophy. Note the arteriole (*arrow*) surrounded by a minor population of deeply basophilic lymphocytes that populate the periarteriolar lymphoid sheath, and the poorly delineated peripheral band of cells (M) that constitute the marginal zone

The spleen exhibits less pronounced age-related histologic changes than the thymus. The periarteriolar lymphoid sheath and marginal zone are histologically discernible in the rat. Follicles, particularly secondary follicles with germinal centers, typically are less numerous and less prominent in aged rats than in young rats (Fig. 5.7a, b). The number of splenic follicles with germinal centers varies substantially

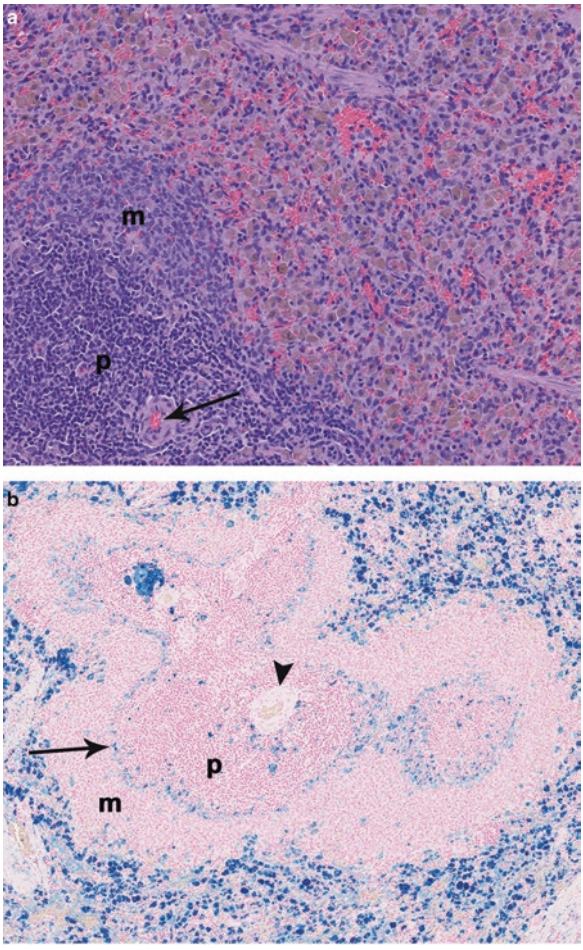

Fig. 5.8 (**a**) The spleen of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has an arteriole (*arrow*) surrounded by periarteriolar lymphoid sheath (p) and marginal zone (m). The nonlymphoid area ('red pulp') has numerous macrophages distended by brown cytoplasmic pigment, consistent with hemosiderin. Hematoxylin & eosin stain, 20× objective magnification. (**b**) The spleen of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has an arteriole (*arrowhead*) surrounded by periarteriolar lymphoid sheath (p) and marginal zone (m). The nonlymphoid area ('red pulp') has numerous macrophages laden with iron-positive pigment, consistent with hemosiderin. Note the concentration of iron-containing macrophages (*arrow*) in the immediate vicinity of the marginal sinus. Perls' Prussian blue iron stain, 10× objective magnification

between individual rats. Histologic cross-sections of the spleen of young adult rats commonly have 3–5 follicles that include germinal centers, but spleen sections of some rats are devoid of follicular germinal centers and other spleen sections may have 15+ follicular germinal centers. While some systemic stimulus to humoral immunity would be anticipated in anticipation with a large number of secondary splenic follicles, in practice that association is rarely documented by the routine pathology evaluation of toxicology studies. Spleen sections of cynomolgus monkeys often have a moderate number of follicles with germinal centers, but follicles and germinal centers are less commonly seen in the spleen of dogs. Histopathologic evaluation of the spleen of dogs is complicated by use of barbiturate euthanasia agents, which result in pronounced blood engorgement of the spleen and associated masking of the fine details of lymphoid structures (*see* Chap. 7, Routine Histopathology).

In aged rats, particularly females, the interfollicular areas ('red pulp') of the spleen commonly have a substantial population of macrophages laded with iron-positive pigment, consistent with hemosiderin (Fig. 5.8a, b).

5.8.4 Histomorphological Effects of Aging on Lymph Nodes

Lymph nodes of aged rats commonly have histologic features of inactivity, including a reduction in the population of primary follicles in the cortex, absence or near-absence of germinal centers, and indistinct or absent high-endothelial venules (Fig. 5.9a, b). Mandibular lymph nodes may retain a prominent population of plasma cells within medullary cords (Fig. 5.10), as is commonly seen in mandibular lymph nodes of young rats. The plasma cell accumulations may be so pronounced that the lymph nodes are grossly enlarged (Fig. 5.11). Subcapsular and medullary sinuses may be dilated (Fig. 5.12), sometimes to the degree that the lymph nodes are overtly cystic upon gross examination (Fig. 5.13). The subcapsular and medullary sinuses often contain histiocytes engorged with finely granular, brown to pink material that consists of hemosiderin and lipofuscin (Fig. 5.14a–c). In some animals the engorged histiocytes are massively enlarged, but have no histologic evidence of proliferation and atypism that would suggest pre-neoplastic or neoplastic transformation. Mast cells may be prominent in lymph nodes of rats of all ages, including aged rats (Fig. 5.15), and are particularly common in the mesenteric lymph nodes (Losco and Harleman 1992).

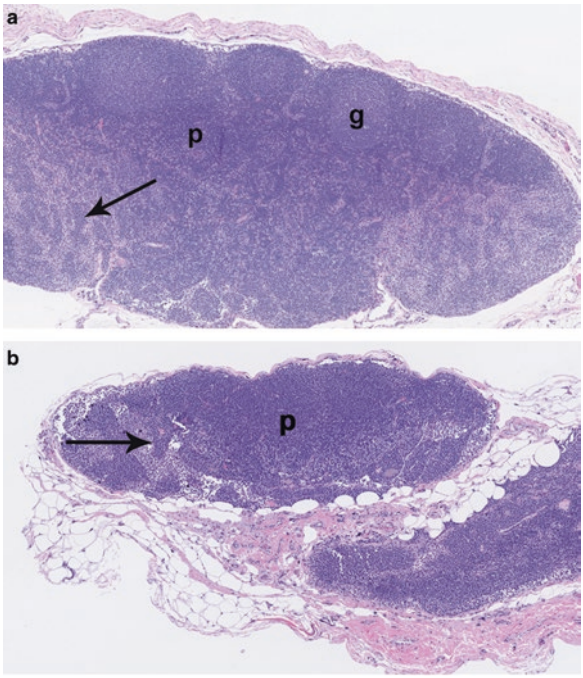

Fig 5.9 (**a**) The mandibular lymph node of a young (postnatal day 42) female Sprague Dawley rat has highly cellular paracortex (p), multiple cortical follicles containing germinal centers (g), and medullary cords (*arrow*) filled with plasma cells and lymphocytes. Hematoxylin & eosin stain, 5× objective magnification. (**b**) The mandibular lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has highly cellular paracortex (p), but lacks the distinct follicles and germinal centers that indicate an active humoral immune response. Medullary cords (*arrow*) are filled with plasma cells. Hematoxylin & eosin stain, 5× objective magnification

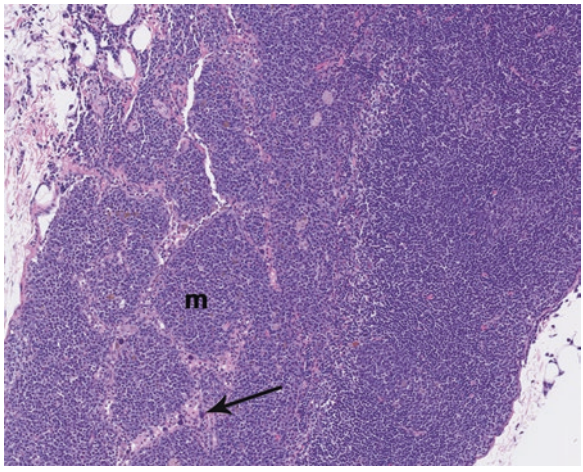

Fig. 5.10 The mandibular lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has medullary cords (m) distended with plasma cells. Histiocytes filled with brown hemosiderin pigment are present in medullary sinuses, and a few deeply basophilic mast cells (*arrow*) are present. Hematoxylin & eosin stain, 10× objective magnification

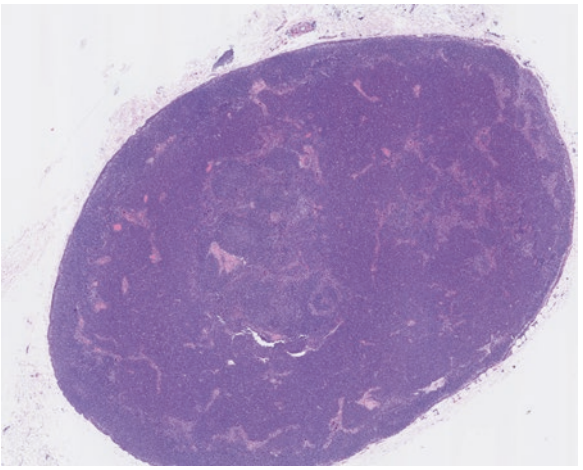

Fig. 5.11 A grossly enlarged mandibular lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has a densely cellular central region that consists of severely dilated medullary cords filled with plasma cells. Hematoxylin & eosin stain, 2.5× objective magnification

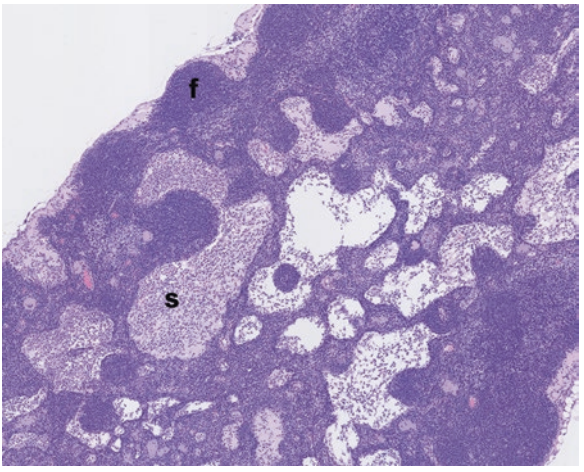

Fig. 5.12 The mesenteric lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has inactive-appearing cortical lymphoid follicles (f) that lack germinal centers. Medullary sinuses (s) are markedly dilated and contain numerous lymphocytes. Hematoxylin & eosin stain, 5× objective magnification

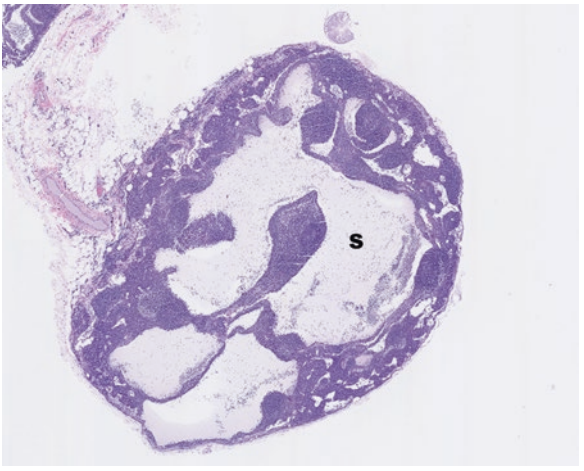

Fig. 5.13 The mandibular lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study was grossly enlarged and cystic at necropsy examination. Histologically, the lymph node has few cortical follicles and severely dilated medullary sinuses (s). Hematoxylin & eosin stain, 2.5× objective magnification

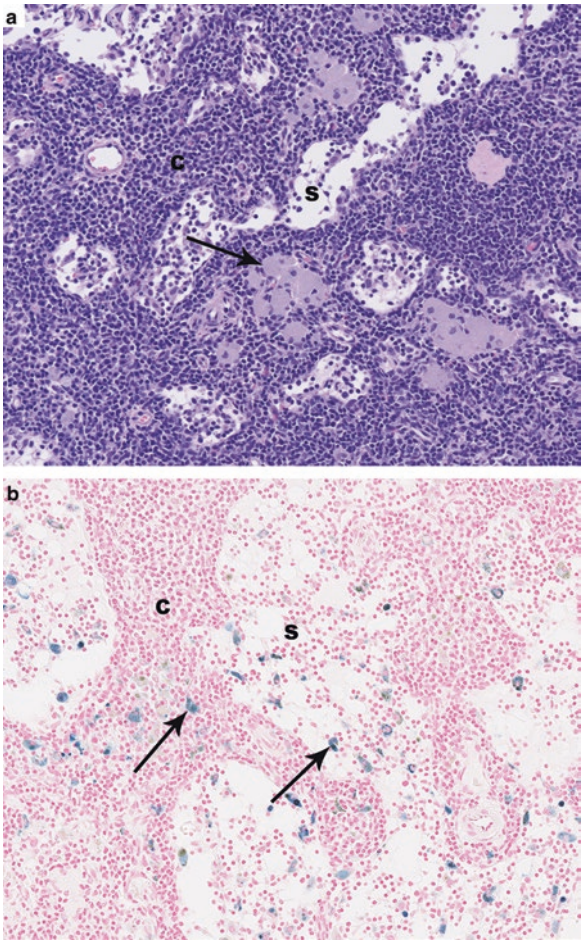

Fig. 5.14 (**a**) The medullary region of a mesenteric lymph node from a male untreated control Wistar-HAN rat collected at termination of a 30-month study has highly cellular medullary cords (c) and mildly dilated medullary sinuses (s). Medullary cords have aggregates of histiocytes (*arrow*) engorged with homogeneous to faintly granular gray-brown material that is of uncertain genesis. Hematoxylin & eosin stain, 20× objective magnification. (**b**) The medullary region of a mesenteric lymph node from a male untreated control Wistar-HAN rat collected at termination of a 30-month study has a moderate number of iron-laden macrophages (*arrow*) located in both medullary cords (c) and medullary sinuses (s). Perls' Prussian blue iron stain, 20× objective magnification. (**c**) The medullary sinuses (s) of a mesenteric lymph node from a male untreated control Wistar-HAN rat collected at termination of a 30-month study have a small number of lipofuscin-laden macrophages (*arrow*). AFIP stain for lipofuscin, 20× objective magnification

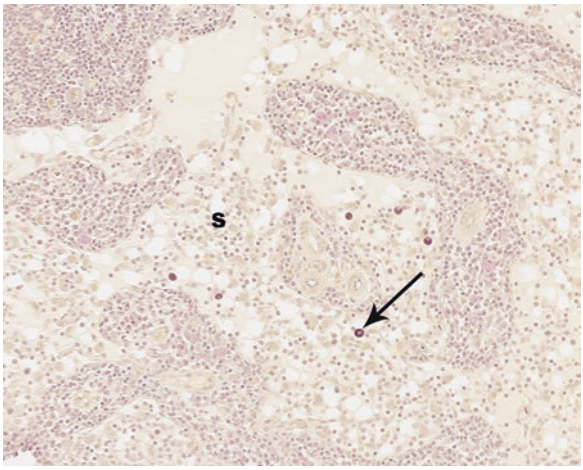

Fig. 5.14 (continued)

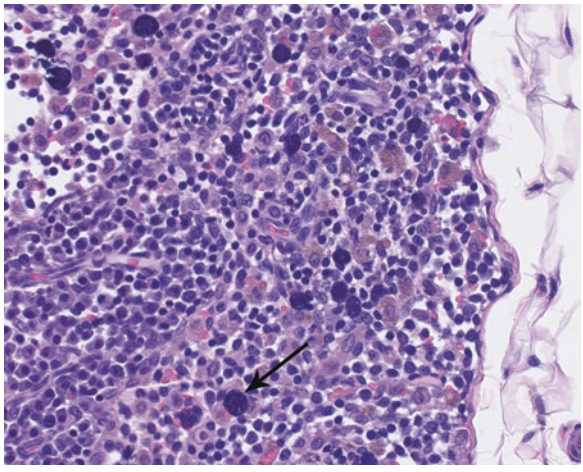

Fig. 5.15 The superficial cortex of the mandibular lymph node of a male untreated control Wistar-HAN rat collected at termination of a 30-month study has a moderate number of densely basophilic mast cells (*arrow*) as well as numerous histiocytes laden with brown pigment. Hematoxylin & eosin stain, 40× objective magnification

5.9 Summary Points

- Aging of the host affects virtually all components of the immune system.
- While the overall effect of aging is a reduction in immune competence, age-related effects most commonly result from dysregulation rather than a direct hindrance or decrement in specific immune system components or functions.
- Aging results in a generalized proinflammatory status in the host ('inflamm-aging'), which predisposes the host to inflammatory disease processes and exacerbates concurrent disease processes such as atherosclerosis, neurodegeneration and osteoporosis.

- Aging results in an increased population of circulating neutrophils which have reduced chemotactic, phagocytic and microbial killing ability, yet retain potential for bystander injury to host tissues.
- Age-related changes in macrophages include reduced phagocytic ability, cytokine and chemokine production, expression of MHC class II, and expression of co-stimulatory molecules such as CD80, thus there is a reduction in macrophage function in both the innate and adaptive immune systems.
- Age-related changes in dendritic cells include reduced migration, pinocytosis, phagocytosis and stimulation of T cell and B cell responses, thus the dendritic cell contribution to responses of secondary immune system organs is diminished.
- Macrophages and dendritic cells have age-related reduced ability to phagocytize apoptotic cell debris, which may then provoke of an inflammatory response that contributes to the generalized 'inflamm-aging' status of aged hosts.
- Circulating NK cell populations increase with age, but an age-related reduction in function of the individual NK cells results in an overall age-related decrement in NK cell activity in the host.
- Age-related thymic involution is associated with a dramatic reduction in the number of naïve T cells exiting the thymus. Subsequent extrathymic T cell generation, which occurs largely in the intestine and biliary tract, lacks the effective selective processes that exist in the thymus, thus the resultant extra-thymically generated T cells have a greater propensity for auto-reactivity. This may contribute to the higher incidence of autoimmune diseases in aged individuals.
- The numerical ratio of $CD4^+$ to $CD8^+$ T cells declines with aging. In addition, there is an age-associated increase in the number of $CD28^-$ T cells that are unable to participate in the CD28/B-7 conjugate that is necessary for T cell activation.
- The major T cell co-stimulatory pathways (CD28/B7 and CD40/CD40L) become dysfunctional with aging.
- An age-related reduction in immunologic diversity in the T_H cell population, in humans resulting largely from T_H cell commitment to various herpesviruses, results in reduced ability to promote immune responses to pathogens.
- Age-related decline in B cell functions is largely based on alterations in T_H cell functions, but B cell-specific changes such as decreased production of B cell precursors, limitation of the B cell immunological repertoire, and accumulation of memory B cells at the expense of naïve B cells, all contribute to the age-related decrement in B cell function.
- The constellation of changes in immune system cell populations and signaling processes results in a reduction in germinal center activity, which is a central event in the generation of humoral immune responses.

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Chapter 6
Flow Cytometry and Immunophenotyping in Drug Development

Tracey L. Papenfuss

Abstract As increasing numbers of novel therapeutics impact immune responses either directly or indirectly, it is necessary to employ techniques to evaluate immune system alterations. Flow cytometry is an important tool that can be applied throughout drug development (e.g. early discovery to clinical monitoring) to evaluate immunobiological and immunotoxicological alterations resultant from therapies. Traditionally, flow cytometry has broad applications in assessing the identity of specific cell populations and their composition within bodily fluids and tissues. Application of cellular phenotype for routine immunophenotyping of cell populations has expanded to include enhanced immuno-phenotyping of cell subsets (e.g. Tregs, Th1, Th2, etc.) and functional immunophenotyping based on unique expression of cytokines, signaling markers and other markers identifying cell populations with specific functions. Although powerful in its ability to determine immunophenotyping, flow cytometry is increasingly being used to interrogate numerous other parameters including, cellular viability (e.g. apoptosis versus necrosis), cellular proliferation, phagocytosis, calcium influence, nucleic acid content, oxidative stress, signaling events and phosphorylation in signaling cascades and numerous others. Instrumentation, reagents and species-specific considerations, sample preparation and preservation, data acquisition, analysis and reporting and validation are all critical considerations in flow cytometry which are covered in this chapter. Ultimately, integration of flow cytometry with numerous other data sets (e.g. immunotoxicology, immunogenicity, pathology, etc.) are critical to develop a comprehensive picture of immune system alterations in drug development which have direction implications on human health.

Keywords Flow cytometer • Fluorochrome • Antibodies • Routine immunophenotyping • Enhanced immunophenotyping • Functional immunophenotyping • Novel therapeutic entities (NTEs) • Cell sorting • Compensation • Isotype controls • Immunotoxicology • Immunotoxicity • Immunomodulation • Helper T cells • Regulatory T cells

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Abbreviations

APC	Allophycocyanin
CD	Clusters of differentiation; e.g. CD4, CD8
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CBC	Complete blood count
DMSO	Dimethylsulfoxide
FMO	Fluorescence minus one
FACS	Fluoroescent antibody cell sorting
FITC	Fluoresceine isothiocyanate
GLP	Good laboratory practice
IHC	Immunohistochemistry
Ig	Immunoglobulin; e.g. IgG, IgM
NTE	Novel therapeutities
MHC	Major histocompatibility complex
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
PMT	Photomultiplier tubes
Th1/Th2/Th17	T helper 1, T helper 2 and T helper 17 cells
Treg	Regulatory T cells

6.1 Introduction

Immune responses are extraordinarily responsive to pertubations from normal physiological processes or the exposure to exogenous agents or xenobiotics (e.g. chemicals, new therapeutics and toxins). Within the realm of drug development, a rapid and robust means to evaluate immunotoxicologic, immunopathological and immunmodulatory effects is critical. In particular, with an increasing number and array of small molecules, novel biologics, vaccines, immunotherapies and other immunomodulatory molecules that either specifically or non-specifically target immune cells, it is critical that the industries engaged in new product development can adequately evaluate the effects of novel xenobiotics on the immune system.

Conventional hazard identification and risk assessment for new agents involves a multifaceted approach that encompasses both functional and structural testing. Functional immune testing traditionally has relied on such in vitro assays, such as cellular proliferation assay, T-dependent antibody responses (TDAR) assays and host resistance assays (Services 2006). The structure-based aspects of immune system evaluations typically are based in routine histopathologic examination or enhanced immunohistopathology of tissue sections from isolated organs, generally including lymph nodes, spleen, bone marrow, and thymus (Elmore 2006a, b,

c, d, e, 2010, 2012). Additional inferences with respect to potential immune responsiveness may be drawn for specific leukocyte classes by clinical pathology evaluation of blood samples for cells of the innate (neutrophils, eosinophils, basophils, natural killer cells, monocytes/macrophages) and adaptive (lymphocytes) arms of the immune system. In recent years, the newer high-throughput tools beyond traditional histopathology and clinical pathology have permitted the more detailed evaluation of leukocyte classes based on the expression of cell type specific markers. The initial application of flow cytometry was in evaluating cellular identify but newer applications in flow cytometry provide a powerful means of evaluating immune cells and responses. This technology has the potential to provide significant value-added and clinical monitoring capabilities in human clinical trials, as well as, the ability to evaluate immune and parenchymal organs in the evaluation of new therapeutic entities (NTEs) in drug discovery and development (Branch and Agranat 2014; Cecic et al. 2012; Dunne and Maecker 2011; Gossett et al. 1999).

Flow cytometry is a simple and rapid method for the quantitative and qualitative analysis of single-cell suspensions (Cecic et al. 2012; Herzenberg and Sweet 1976; Raveche et al. 2011; Pedreira et al. 2013). Flow cytometry is a laser-based technology whereby cells or other cellular or biophysical components are suspended in a fluid stream and stimulated by a laser, after which they scatter and/or emit light which is then detected by photomultiplier-based tubes (PMTs) (Cecic et al. 2012; Pedreira et al. 2013). Modern flow cytometers are now combining principles of optics, fluidics and electronics to better detect cell type-specific markers that have functional significance (Coulter 1953; Herzenberg and Sweet 1976; Hulett et al. 2014; Smith et al. 2011).

These technologies can be rapidly brought to bear on drug development applications when the need arises, assuming proper instrumentation and trained personnel are available. However, although technologically advanced modalities such as 17-color, high dimensional comprehensive leukocyte immunophenotyping (CLIP) and even cell phone-based flow cytometric evaluation of intracellular phosphorylation (e.g. JAK-STAT signaling) analyses may be available, such applications may be of variable utility within the drug development arena and are more commonly used within an academic setting (Biancotto et al. 2011; Chattopadhyay et al. 2004; Chattopadhyay et al. 2010; Perfetto et al. 2004; Baan et al. 2012; Perez et al. 2004).

A significant amount of information can be obtained from flow cytometry data, depending upon the application of interest, sample labeling strategy, and instrumentation. Cells, cellular components (e.g., microvesicles) or particles (e.g., microparticles) can be evaluated based on their size (via “forward scatter” of light) and granularity (by “side scatter” of light), but additional information is typically acquired through the application of fluorescent labels (or fluorochromes) that can be excited and subsequently detected in the cytometer. Fluorochromes are most commonly conjugated to antibodies that recognize and bind the molecule of interest. Light emission by the fluorochrome excited laser thereby signals the presence

of a cell or cell part that expresses the marker. Commonly employed fluorescent probes include fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC). The typical nomenclature for a fluorochrome-conjugated antibody is as follows: rat anti-mouse CD4 IgG_1-FITC, which indicates that an IgG_1 antibody generated in a rat against mouse CD4 has been conjugated to FITC. Although FITC, PE and APC are standard agents used to label antibodies, a large array of newer fluorochromes, tandem dyes, and other new technologies such as quantum dot semi-conductor nanocrystals make 17-color flow cytometry to simultaneously detect multiple cell classes and functional states a reality (Cecic et al. 2012; Chattopadhyay et al. 2010; Chattopadhyay et al. 2006; Perfetto et al. 2004). While labeled antibodies to cellular targets probably constitute the most common application of flow cytometry, a number of commonly used flow cytometric analyses involve non-immunological molecular interactions. In fact, nearly any molecule that has fluorescence capabilities (e.g., with distinct excitation and emission spectra) can be utilized for detection by flow cytometry. Such "non-specific" fluorescent probes can be used for a variety of applications including cell proliferation (e.g., carboxyfluorescein diacetate succinimyidyl ester; CFSE), cell viability/death/apoptosis (propidium iodide; PI, 7-Aminoactinomycin D; 7-AAD or Annexin V/phosphatidylserine), phagocytosis of fluorescently-labeled beads or target pathogens, calcium influx (using the Ca^{++} indicator fluo-3-acetoxymethyl ester) and a host of other applications. Monoclonal antibodies directly conjugated to the desired fluorochrome are most commonly employed, and these are typically species-specific. A large and sophisticated industry has evolved to meet the reagent needs of flow cytometry applications. However, species-specific, directly conjugated antibodies may not be available or even desirable for some applications, which may necessitate the use of secondary or additional antibodies to identify the cellular marker of interest.

Although flow cytometry was first used to identify individual populations of cells, its application has expanded into detailed interrogation of cell functionality and the ability to interrogate subcellular and non-cellular components as well. A detailed discussion of all of the potential opportunities applications is beyond the scope of this chapter, but some opportunities relevant to drug development are outlined in Table 6.1 Immunophenotyping is perhaps the most recognized routine application of flow cytometry for immune system evaluation whereby the percentage of cells, immune cells and subcategories of T and B lymphocytes or other immune cells are determined in blood samples (Cecic et al. 2012; Lappin 2010; Raveche et al. 2011). Immunophenotyping can be applied during all stages of drug discovery and development. However, the marked advances in recent years that allow very detailed interrogations of cell type-specific phenotypic subsets, activation status and functional parameters are of particular utility in the early stages of drug development. These include evaluating mechanisms of cell death (necrosis versus apoptosis), cell cycle components, nucleic acid content, oxidative stress, calcium flux, membrane potential, and the presence of specific transcription factors and cell signaling events, phosphorylation of signaling moieties on tyrosine

Table 6.1 Applications of flow cytometry in drug discovery, target validation, toxicology and clinical testing during drug development

		Assays
Drug discovery and target validation	Assessment of cell toxicity (viability and physiological state)	Cell size
		Cell growth and death rates
		Cell cycle and nucleic acid content
		Apoptosis
		Oxidative stress
		Calcium flux
		Efficacy (functional) assays
		Cell signaling
	Characterization of binding properties and receptor occupancy of biologics	
	Pharmacokinetics	
Toxicology	Ex vivo studies in BM, SPL, LN, peripheral blood	
	Assessment of effects on hematopoiesis	
Clinical testing	Cell phenotyping	Lymphocyte immunophenotyping
		PD biomarker assessment
		Endothelial cells
		(Circulating metastatic) Tumor cells
		CD34 stem cells
	Functional assays	HLA specific tetramers for T cell responses
		Intracellular cytokine production
		Intracellular signaling
		Proliferation responses
	Pharmacodynamics	Functional assays
	Immunogenicity assays	Detection of ADA neutralizing Abs

residues, etc. (Burchiel et al. 1999; Burns-Naas et al. 2007; Krutzik et al. 2011; Krutzik et al. 2004; Lappin 2010; Perez et al. 2004; Sklar et al. 2007). Identification of rare cell populations and specific biomarkers, as well as, the ability to sort and collect these cells are very powerful tools to ascertain very subtle effects on components of the immune system (Baron et al. 2012; Cecic et al. 2012; Sklar et al. 2007). Importantly, the selection of instrumentation should take into consideration the current and future needs since cell sorting can only be a accomplished with a flow cytometer with sorting capabilities. Additional applications in pre-clinical drug development include imaging cytometry, phospho-specific drug screening, receptor pharmacology and many others (reviewed in (Green et al. 2011)).

6.2 Uses of Flow Cytometry in Toxicology

6.2.1 Early Discovery

Within the early discovery arena, elucidating the effects on immune system function can help determine "go" or "no-go" decisions early in the pipeline and prior to moving a potential therapeutic forward into safety testing. The ability to interrogate not only mechanisms of immune function in health and disease but also to assess mechanisms and efficacy of various test articles (ranging from adjuvant and vaccine efficacy, cell-based immunotherapy and novel drug, to small molecule and biologics) provides significant value in early phases of NTE evaluation, which may help refine the choice of disease indications toward which the agents may be targeted. Flow cytometry is often used during drug discovery and target validation to help define mechanisms of action, pharmacokinetics, genomic/proteomic screening, cell target detection/specificity, drug hit-to-lead generation, lead optimization, receptor binding/occupancy and general assessment of cell toxicity (Lappin 2010; Lappin and Black 2003; O'Hara et al. 2011; Owens et al. 2000; Sklar et al. 2007; Xu and Richards 2011). In determining receptor occupancy and pharmacokinetics, flow-based assays may be an ideal platform to measure the level of receptor engagement on cell surface or receptor occupancy and may indeed be superior to plate-bound assays since epitopes/receptors in suspensions of fresh (i.e. unfixed) cells are in their native form (DeSilva et al. 2003; O'Hara et al. 2011; Xu and Richards 2011). Cellular phenotyping can evaluate potential adverse events such as overt immunotoxicity early in discovery. Lymphopenia can be a relatively common manifestation of immunotoxicity and/or supportive of functional immunosuppression but routine evaluation fails to identify which lymphocytes are primarily depleted. Flow cytometry can identify whether there is select loss of CD8+ or CD4+ T cells and enhanced immunophenotyping can even identify potential loss of subpopulations (e.g. CD4+ Th1, Th2, etc. populations) Additionally, the application of more advanced cellular immunophenotyping (e.g. T cell sub-classes) or "functional" phenotyping of various cell populations can be employed to gain an understanding of more subtle effects such as immune activation or immune suppression. Detection and evaluation of intracellular and secreted cytokines can be a very powerful tool to evaluate not only the functionality of the various leukocyte subsets (i.e. whether they are immunostimulated/inflammatory or immunosuppressed/regulatory) but also the relative degree of immune activation or exhaustion (Freer and Rindi 2013; Pala et al. 2000). Data obtained from earlier discovery phases of drug development can further be applied and adapted during transition from nonclinical to clinical testing where changes in morphologic and/or functional aspects of target cells can be measured and monitored and dosages can be determined (Owens et al. 2000; Visich and Ponce 2008).

6.2.2 Safety Assessment and Toxicology

Within the toxicology and safety arena, flow cytometry has been primarily used to determine the effects of the test article on composition and alterations in various immune cell populations. Blood samples are most commonly evaluated in toxicology studies although ex vivo evaluation of both immune (e.g. bone marrow, spleen, thymus, lymph nodes) and non-immune (e.g. tumor-specific cells, skin, etc.) organs can be evaluated. With a knowledge and application of lineage-specific markers, a robust set of data on the toxic effects of a test article on various hematopoietic lineages can be determined using routine flow cytometry. Standard panels of antibodies are routinely applied to blood samples in toxicology studies where the percentages of T cells, natural killer (NK) cells, granulocytes and myeloid cells can readily be determined. Additionally, definitive enumeration of various hematopoietic precursors can accurately identify the primary target in bone marrow toxicity (Reagan et al. 2011). Increasingly, specific cell subsets (e.g. regulatory T cells), alterations of overall immune responses (e.g. Th1, Th2 and Th17 responses) and/or functional immunophenotyping and immune parameters of specific populations are being applied during safety/toxicology studies. Throughput is often the limiting factor preventing the routine application of flow cytometry in preclinical toxicology, particularly on samples other than blood (Cornelissen et al. 2012; Le Meur et al. 2007; Sklar et al. 2007). However, as protocols for processing major immune organs have become more standardized, the application of flow cytometry for carefully designed animal studies is gaining in stature as an important means for in vivo examination of immune system integrity.

6.2.3 Clinical Testing and Monitoring

A major goal of preclinical testing of therapeutic products is identification of reliable biomarkers of toxicity for use in clinical trials. As in other stages of drug development, continual monitoring of immune effects during human clinical trials can be accomplished using flow cytometric immunophenotyping. This strategy typically is undertaken using blood samples, although in special instances cells harvested from biopsy of a peripheral lymph node may be utilized instead. This analysis can provide valuable insight into the impact of treatment on patient populations as well as identify and monitor cellular or other biomarkers indicative of therapeutic efficacy or toxicity (Hedley et al. 2008; O'Gorman and Zijenah 2008; Owens et al. 2000; Vesterqvist and Reddy 2011) Flow cytometry can be used in early phase clinical trials to translate a biomarker identified in nonclinical studies for use in human trials (Hedley 2011; Litwin and Andahazy 2011). Functional flow cytometric assays such as the use of HLA-specific tetramers for evaluating T cell responses and evaluation of intracellular cytokine or signaling events can provide critical monitoring information of immune responses in patients. Flow cytometry can also assist in

determining adverse effects such as immunogenicity and the development of anti-drug antibodies, which is an increasingly common challenge with the increasing number of biologics being explored for the treatment of disease (Freer and Rindi 2013; O'Hara and Theobald 2011; O'Hara et al. 2011; Pala et al. 2000). The decisive application and marked array of additional assays potentially available with flow cytometry technology provides a powerful tool to interrogate the immune system in a very detailed and elegant manner during all stages of drug development.

Immunophenotyping is the analysis and identification of the cell populations of interest within a larger heterogeneous population based on specific identifying (phenotypic) characteristics. In flow cytometry, it typically is based on the relative expression of cell surface markers on various cell types. Routine antibody panels evaluating populations and subpopulations of T cells, B cells and NK cells are most commonly evaluated in animals and humans, but additional cell populations can be evaluated based on the availability of species-specific antibodies. Typical panels probe lymphocyte subsets (e.g., CD3, CD4, CD8, NK/NK-T cells, B cells); some of the more commonly used antibodies for rats, dogs and non-human primates are listed in Table 6.2. Although academic research institutions may utilize 17-color analyses of subsets or comprehensive leukocyte immunophenotyping (CLIP), these strategies are less commonly employed in the drug development setting due to limitations with or lack of instrumentation, data analysis software , trained technical personnel, and/or experts capable of fully analyzing and communicating these data sets (Biancotto et al. 2011; Chattopadhyay et al. 2006; Perfetto et al. 2012; Perfetto et al. 2004).

Enhanced and functional immunophenotyping are not routinely employed in drug development but are of increasing interest in this setting given the significant increase of drugs, therapies, biologics and other entities which target the immune response either directly or indirectly either as part of the therapeutic application or as an adverse side effect. Enhanced immunophenotyping extends the traditional method to include additional populations (e.g. monocytes/myeloid cells, granulocytes), subsets of populations (e.g. NK-T cells, Th1/Th2/Th17/Tregs, M1/M2 macrophages, etc.) and the degree of maturation/differentiation (e.g. B cell precursors versus plasma cells). Functional immunophenotyping evaluates cells or populations of cells for their relative functional status using flow cytometry directly on ex vivo samples or on clinical samples that have been cultured and treated (e.g. stimulated) in vitro for specific timeframes using a particular antigen or stimulus. Such functional assays can include determination of non-specific characteristics like cellular activation status (e.g. CD80/CD86 on myeloid cells, CD25/CD69/CD49 on T cells, etc.), proliferation (CFSE or other dye-based assays), cell cycle kinetics, cell death and the type of death (e.g. apoptosis/necrosis/necroptosis), calcium utilization, and DNA content. Other functional assays are suited to evaluation of such cell population-specific functions as cytokine production, cytotoxicity, oxidative burst activity, biomarker expression (e.g., antigen-specific tetramer-specific T cells), and others. Figure 6.1 outlines some examples of possible routine, enhanced and functional immunophenotyping that may be performed. Specifically, flow-based evaluation can complement numerous other *in vitro* immune assays that assess cellular immune responses (Fuggetta et al. 2009).

Table 6.2 Panel of some commonly employed immunophenotyping panels in studies for rat, canine and non-human primate studies

	Rat	Canine		Non-human primates
T cell	CD3+ (CD5+, CD2+, CD6+)	CD5+ OR	CD3+	CD3
T helper cell	CD3+CD4+	CD5+CD4+	CD3+CD4+	CD3+CD4+CD8-
Treg cells	CD45RClo, CD4+CD25+FoxP3+	CD4+CD25+FoxP3+		CD4+CD25+FoxP3+
T cytotoxic cell	CD3+CD8+	CD5+CD8+	CD3+CD8+	CD3+CD4-CD8+
B cell	CD3-CD45RA+	CD5-CD21+	CD3-CD21+	CD3-CD20+ CD20+CD8- CD20+CD8+ CD20+CD1c- CD20+CD1c+
Monocyte	CD3+CD161a+	CD5-CD14+	CD3-CD14+	CD3-CD14 HLA-DR+CD14+CD16- HLA-DR+ CD14+/−CD16+
pDCs	MHCII+CD11b-OX62-	N.D.		pDC CD123+
DCs	CD172a+,MHC-II+ CD11c+ alphaE2 integrin+	CD11b+CD11c+		mDC1 CD11c+CD16- mDC2 CD11c-CD1c+ CD205+ CD141+ (of variable utility)
Macrophages	CD172a+,MHC-II+ CD68+, CD11c+	CD11b+CD11c-		CD68+ CD163+
NK cell	CD3-CD161a+ NKR-P1A	N.D.[a]		CD3-CD16+ CD16+CD8+CD20- CD16-CD8+CD20−/dim
NKT Cells	CDC3+CD161a+	N.D.[a]		CD3+CD16+

[a]Suggested (MHC II+CD11/18+, CD56+)

6.3 Considerations in the Implementation of Flow Cytometric Analysis

6.3.1 General Considerations

Given the significant variables involved in flow cytometric analyses, the Centers for Disease Control (CDC) categorizes flow cytometry as a high complexity laboratory test (Wood et al. 2013) (reviewed in (Owens et al. 2000)). These variables, which include complex instrumentation, lack of cellular references, a plethora of validations and controls, and the combination of cells and reagents, make flow cytometry more challenging to validate compared to other methodologies used in drug development. When used to support drug development, detailed protocols subjected to

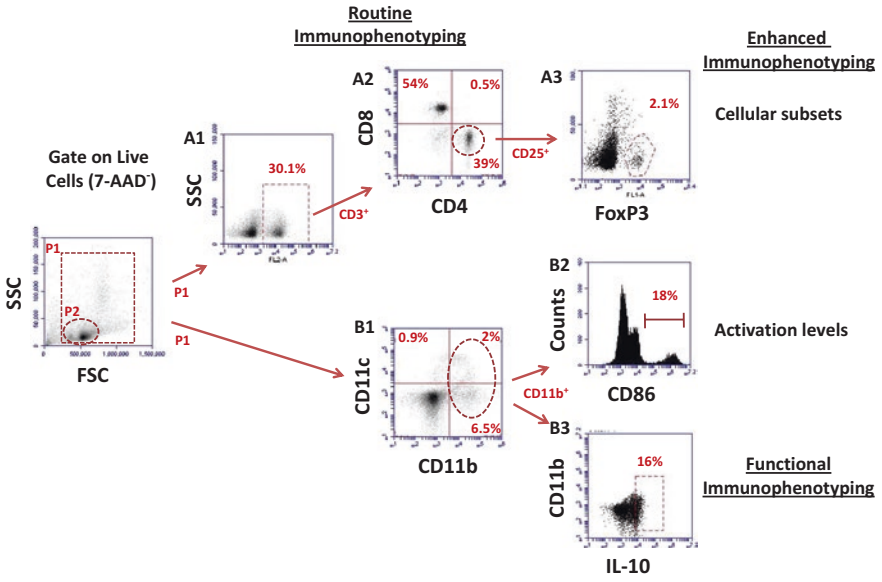

Fig. 6.1 Examples of data from mouse samples showing commonly employed routine, enhanced and functional immunophenotyping panels by flow cytometry. Cells are collected and size and granularity are evaluated by FSC and SSC. Processed and stained cells can then be analyzed for expression of various markers. Often, non-viable cells can be excluded by gating out viability dyes, such as 7-AAD. Cells of interest can be selected from a particular gate. In Fig. 6.1, P1 outlines viable cells and excludes cellular debris while P2 outlines the typical location of mouse lymphocytes. The upper (*A*) panels indicating subsequent gating from the FSC/SSC gating, followed by *A1*) gating on CD3+, followed by subsequent gating (*A2*) to determine routine immunophenotyping of CD3+CD4+ or CD3+CD8+ cell populations. Further gating in combination can provide enhanced immunophenotyping of specific subsets. Additional staining and gating (e.g. CD25+ and FoxP3) can identify (*A3*) the percentage of CD4+CD25+FoxP3+ regulatory T cells. The lower panel also demonstrates routine immunophenotyping to provide (*B1*) percentages of dendritic cells (i.e. CD11c+ in mice) versus macrophages/other additional CD11b+ myeloid cell populations. In additional to enhanced immunophenotyping which can provide information regarding (*B2*) activation status (e.g. CD86), additional functional immunophenotyping including (*B3*) cytokine production (e.g. IL-10 production in CD11b+ cells) can be ascertained. These examples provide only a handful of immunophenotyping capabilities potentially available with various staining and gating combinations. Samples were obtained from mouse spleens and cells analyzed using an Accuri C6 cytometer

both rigorous optimization and validation should be performed for each assay of interest. In general, flow-based assays should examine multiple immune cell classes, including elements of both the innate and acquired arms of the immune system. A detailed description of the various requirements and protocols for appropriate sample preparation and analysis of immune organs by flow cytometry are beyond the scope of this chapter, but such information is available in many other publications. In the following sections (outlined in Table 6.3), some considerations are given for instrumentation, antibody selection, sample preservation and processing, assay validation, and data management. All of these variables must be taken into account and often validated empirically with each sample type, selected antibody, instrument, and data analysis protocol.

Table 6.3 Considerations for flow cytometry applications in drug development. A list of potential considerations for sample type, sample preparation, staining, panel/antibody selection and data analysis are listed

Sample type	Sample preparation	Staining	Panel/antibody selection	Data analysis
Blood	Anti-coagulants	Species-specific/cross-reactive antibodies	Available antibodies	Instrumentation
Aspirate	Erythrocyte lysis	Fc blocking with antibodies or serum	Emission/excitation characteristics	Time between storage/analysis
Biopsy	Collagenase to disrupt tissues	Staining order (surface/internal markers)	Spectral overlap	Digital/analog compensation
Bone marrow	Need for specific buffers, fixatives	Cross-reactivity between antibodies	Antigen-antibody saturation	Isotype controls
Tissue samples		Fixation and fixative	Optimal signal-to-noise ratio	Isoclonic controls
		Permeabilization	Minimize background fluoresences	Positive/negative controls
			Antibody specificity	Bead-based compensation
			Steric hindrance	Analysis software
			Multiple epitopes	

Table 6.4 Listing of some of the currently available flow cytometry instruments. Additional cytometers may be available and older instruments currently in use for drug development applications may not be included. Cytometers are organized by laser numbers and potential colors available. Table 6.4A lists some potential intrumentation options for various applications

Lasers (max #)	Colors (max)	System	Company	Additional capabilities/features
1	1	MOXI Flow™	ORFLO Technologies	
		Muse™ Cell Analyzer	EMD Milipore	
	3	Guava easyCyte™ 5 and 5HT	EMD Millipore	
		NovoCyte™ 1000	ACEA Biosciences	
2	**3**	CyFlow® Cube Sorter	Sysmex Partec	Sorting (FACS)
	4	BD Accuri™ C6	BD Biosciences	
		BD FACSCalibur™	BD Biosciences	Sorting (FACS)
		CyFlow® Cube 6	Sysmex Partec	
		Guava easyCyte™ 6-2L & 6HT-2L	EMD Millipore	
		NovoCyte™ 2000	ACEA Biosciences	
		S3™ Cell Sorter	Bio-Rad	Sorting (FACS)
	6	Guava easyCyte™ 8 and 8HT	EMD Millipore	
		NovoCyte™ 2060	ACEA Biosciences	
		SE520EXi	Stratedigm	
3	8	BD FACSVerse™	BD Biosciences	
		BD FACSCanto™ II BD	Biosciences	Hydrodynamic focusing
	9	A50-Universal	Apogee	
		CyAn ADP Analyzer	Beckman Coulter	
	10	Guava easyCyte™ 12	Amnis (EMD Millipore)	
	13	CytoFLEX	Beckman Coulter	
		NovoCyte™ 3000	ACEA Biosciences	

4	6	BD FACSJazz™	BD Biosciences	Sorting (FACS)
		CyFlow® Cube 8	Sysmex Partec	
	10	FloSight	EMD Milipore	Imaging
		Gallios™	Beckman Coulter	
	14	Attune® NxT	Life Technologies	Acustic focusing
	18	BD LSRFortessa™	BD Biosciences	
		S1000EXi	Stratedigm	
5	**13**	CyFlow® Space	Sysmex Partec	Sorting (FACS) option
	18	BD LSRFortessa™ X-20	BD Biosciences	
6	**18**	BD FACSAria™ III	BD Biosciences	Sorting (FACS)
		BD FACSAria™ Fusion	BD Biosciences	Class II Type A2 biosafety cabinet Sorting (FACS) option
7	**10**	Amnis ImageStream^X Mark II	Amnis (EMD Millipore)	Imaging
10	**22**	BD Influx™	BD Biosciences	Sorting (FACS)

Specific applications				
	Application	System	Company	Capabilities
	HIV/AIDS	Auto40	Apogee	$CD4^+$ counts
		CyFlow® miniPOC	Sysmex Partec	$CD4^+$and$CD4^+$%
		CyFlow® Counter	Sysmex Partec	WBC, $CD4^+$ and $CD4^+$%
		BD FACSCount™	BD Biosciences	$CD4^+/CD8^+/CD3^+$ T-cell counts
	DNA content	CyFlow® Ploidy Analyser	Sysmex Partec	
	Small particles	A50-Micro	Apogee	Particles 10–100 μm diameter

6.3.2 Instrumentation

All flow cytometers rely on the basic principle of a fluid stream bearing a sample of interest, excitation or interrogation by a laser and detection of the emitted light in a particular pattern. With advances in technology, instrumentation that had originally required a large spatial footprint can now fit on a benchtop and be readily transported. Many flow cytometers are available and utilized for nonclinical and clinical toxicology studies. Table 6.2 provides a list of some of the currently available instruments and recommendations guiding equipment selection and use (Chapman 2000; Green et al. 2011; Smith et al. 2011; Zucker and Chua 2010; Zucker and Fisher 2013; Zhu and Ozcan 2013; Zhu and Ozcan 2015). Some of the most important considerations when contemplating instrumentation include the number (and type) of lasers and the number of available channels, which equate to the number of colors that can be evaluated simultaneously for a single sample.

Additional technologies such as the ability to sort (collect) cells during flow cytometric evaluation and image acquisition are very powerful additional attributes of modern platforms. Cell sorting allows a population of cells to be removed during data capture and collected for more detailed interrogation by other techniques, including other flow cytometric applications. Image acquisition is a recent addition to the flow cytometry field which permits confocal-type technology to capture images of cells within the fluid stream of a cytometer. These newer attributes augment the amount of information in the final data set beyond the simple quantification of various immune cell lineages that is provided by conventional immunophenotyping techniques.

The additional features available on modern flow cytometers, such as with cell sorting and multi-color detection capabilities, can add a substantial cost for equipping the laboratory. These costs associated with the acquisition and maintenance of such instruments can be significant and may be beyond the budget of many smaller organizations or companies. Typically, larger industrial firms or academic institutes have common core facilities that support such cell sorting capabilities on a regular basis, and increasingly partnerships are being developed between academia and industry that will permit the expanded use of this technology for drug discovery and development in the future.

Relative to the instrumentation, personnel needs and qualifications are often given less consideration in the application of flow cytometry. However, the availability of well trained and experienced staff in suitable numbers is equally, if not more, important in the appropriate application of flow cytometry in immunophenotyping and other applications (Chapman 2000). Specifically, availability of a proficient flow cytometry technician who can acquire and analyze flow cytometric histograms can mean the difference between robust data sets versus more variable and thus relatively useless data. The best vendors will provide support not only for the instrument and specific applications of importance to pharmaceutical applications but also will offer basic and advanced training for the individuals charged with utilizing them.

6.3.3 *Sample Collection and Processing for Immediate Flow Cytometric Analysis*

Preparation of single-cell suspensions is a basic requirement for evaluating samples by flow cytometry. Ideally, samples are prepared and immediately analyzed by flow cytometry (i.e. within12–24 h). This is relatively easy to accomplish with blood or other fluid-based samples containing cells which may require only erythrocyte lysis and enumeration of total nucleated cell numbers. Sample collection and analysis of tissue or non-fluid samples, however, requires additional planning and preparation. A basic diagram of some standard sample considerations and basic protocols for preparing samples for flow cytometric analysis are outlined in Fig. 6.2. Standardizing and validating sample preparation for each tissue of interest is strongly encouraged (or required) since different tissues require different handling and preparation techniques. For example, single-cell suspensions can be easily obtained from many primary and secondary lymphoid organs (e.g. thymus, bone marrow, spleen and lymph nodes) from smaller species by manual dissociation but may require additional (enzymatic) tissue dissociation for larger species due to the often increased amounts of connective tissue in these organs. Tissue dissociation (also referred to as

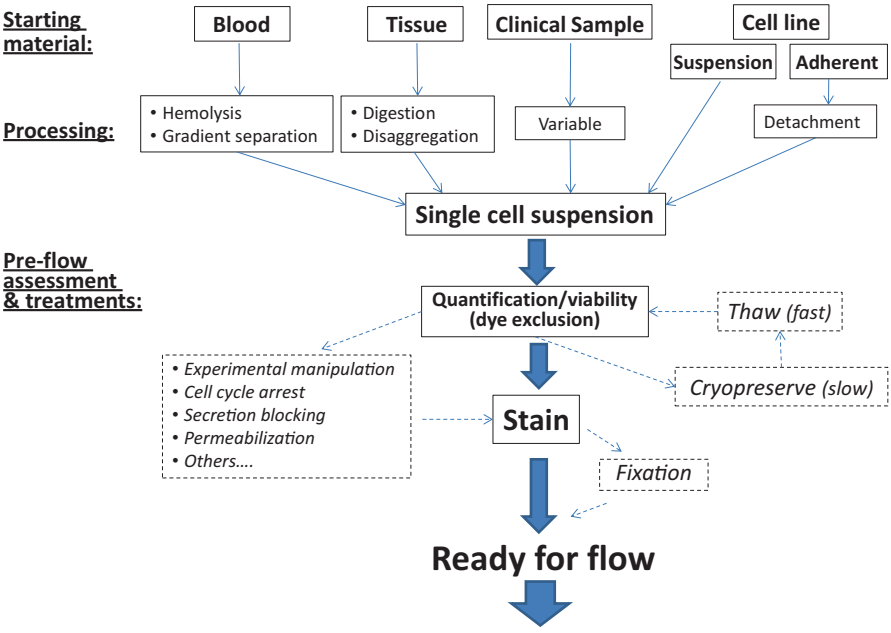

Fig. 6.2 Sample preparation considerations for flow cytometry. Some basic preparation requirements and general processes for samples and tissues are listed. Procedures which can be implemented depending on the application and protocol are provided in dashed boxes. The reader is encouraged to refer to the text and reference publications specific to the potential application and sample of interest

"digestion") is also commonly required for evaluated other non-lymphoid tissues with increased stromal connective tissue (e.g. lung, liver, kidney, tumors, etc.). Any tissue-based flow cytometry samples are likely to have increased cellular debris and/or bone fragments (if from bone marrow) which often must be filtered out prior to running these samples within the cytometer. Standardized operating procedures (SOPs) which outline all aspects of sample origin, preparation, volume, cell density for staining and antibody concentrations are highly recommended as these will directly impact data collection and interpretation. Additionally, evaluating a consistent density of cells within a flow cytometric samples minimizes damage or time lost to instruments which may be clogged by high-density samples, or time lost due to low-density samples which would require longer collection times to achieve a sufficient number of data points or "events" for analysis.

Proper tissue handling, preparation, processing and storage are of paramount importance to ensure confidence in flow cytometric findings. Incorrect handling or storage of samples can quickly result in difficulties which directly impact the validity of the results, therefore, established protocols and built-in quality control points within the processing greatly enhance the confidence in positive and negative findings. In-life studies can be a particularly amenable to the incorporation of flow cytometric evaluation. For example, immune cell phenotyping can be readily performed on blood samples that are already collected for routine hematological analysis and provide additional information regarding the identify and relative percentages of various immune cells. Flow cytometry can be useful as an adjunct to existing diagnostic evaluation (e.g. complete blood count, cytologic bone marrow evaluation, etc.) or as a stand-alone for blood, tissue or other fluid-based samples (e.g. cerebrospinal fluid, bronchoalveolar lavage, peritoneal lavage, etc.). While lymphopenia can be assessed in a complete blood count (CBC), relative shifts in lymphocyte populations and maturity can be assessed by flow cytometry and may be the first subtle in vivo indicator of immunotoxicity. Additionally, relative levels of specific subpopulations (e.g. regulatory T cells; T_{regs}) can be determined by flow cytometry and can be used to evaluate alterations in immune function, as biomarkers of disease course and potential response to therapy in disease models or clinical studies. Cytometric evaluation of biopsy samples may provide important test article-related effects that can be used to inform later stages and study endpoints. Such data can provide important clues regarding mechanisms of actions and/or toxicity and incorporating flow cytometry into an existing experimental design often provides significant value-added. However, prior planning in experimental design and sample collection is critical. While many flow-based assays can "piggy-back" on existing samples such as blood collected for a CBC, there may be specific requirements in flow-based protocols that necessitate separate and/or additional sampling for flow-based assays.

6.3.4 Advanced and Enhanced Sample Preparation

Advanced applications in flow cytometry often require additional and specialized reagents and protocols, all of which must be developed and validated in each respective lab. Applications evaluating intracellular markers (e.g. detection of transcription

factors, intracellular cytokine production, etc.) often require permabilization with reagents such as saponin or other detergent molecules. Often, these may be used in conjunction with other reagents, such as when intracellular cytokine detection is desired. For example, intracellular cytokine detection requires not only the permeabilization to detect the cytokine of interest, but requires sufficient incubation time and a means to accumulate sufficient cytokine for detection (e.g. brefeldin or monensin which block cytokine secretion). Secreted cytokine can also be detected by the immediate capture of cytokine upon secretion (e.g. cytokine bead array) or detection of soluble cytokines themselves within a liquid sample by multiplex available through various vendors. For any of the advanced applications, a thorough understanding of not only the flow cytometric aspects, but also of the techniques involved, impact of cellular biology and desired data read-outs are all considerations that should be identified in advance and optimized prior to use in a large study.

6.3.5 Sample Preservation and Storage for Delayed Flow Cytometric Analysis

Immediate evaluation may not be feasible if large numbers of samples are generated or if samples are shipped for flow cytometric analysis. In these and other cases where immediate analysis is not feasible, an important consideration is proper preparation and storage for later analysis. However, it should be noted that any preservation or storage may impact the resultant data endpoints and that significant optimization, validation and comparison against fresh sample data should be incorporated whenever possible. Samples can often be kept for 24 h without fixation prior to analysis but the specifics (e.g. storage temperature, suspension media, etc.) should be determined for each antibody or antibody combination. Similarly, short-term storage (<7 days) has been shown to be feasible without loss of fluorescence signal but the protocols for each sample type, preparation and storage conditions must be determined and validated prior to study. Some vendors may provide suggested protocols and/or products (e.g. http://www.stemcell.com/en/News/82a6b/March-Tech-Tip-Fridge-Freeze-or-Fix.aspx) (Brown et al. 2015). Fixation of tissue samples or suspended cells can be helpful to allow access for further interrogation but the stability and type of fixative and ability to detect markers in fixed cells must be validated, as marker/antibody integrity and autofluorescence may change dramatically following fixation (Brown et al. 2015; Davis et al. 2011; Pala et al. 2000; Stewart et al. 2007).

Freezing samples is not necessarily an ideal as a means of preserving immune cells for flow cytometric analysis, but is often commonly employed due to the extensive time required to complete all aspects of tissue collection, preparation, staining and data collection. In such instances, freezing of samples following tissue collection and single cell suspension preparations may be preferred as these samples can be analyzed later or fixation would impact the ability to detect certain markers of interest (Pinto et al. 2005; Winter et al. 2014). If necessary, collected tissues must be promptly and processed to single cell suspensions with an appropriate cryoprotectant to maintain cell (and cell marker) integrity. This protocol can be accomplished by using 50% dimethylsulfoxide (DMSO) mixed with 50% serum

(e.g. fetal bovine or species-specific serum) or, alternatively, with commercially-available proprietary buffers. Gradual freezing in freezing containers and protocols designed to control the rate of freezing (e.g. Mr. Frosty) is preferred due to the diminished cell death in cells upon thawing. Frozen cells may be a preferred sample for some applications ranging from early discovery (e.g. pharmacodynamic responses, to clinical monitoring of immune effects (e.g. evaluation of antigen-specific T cells with major histocompatibility complex [MHC] multimers, functional/phenotypic characterization of human peripheral blood mononuclear cells (PBMCs), etc.) (Wyant et al. 2008; Hadrup et al. 2015; Weinberg et al. 2009; Weinberg et al. 2010). Ideally, the initial preservation to consider for immunophenotyping projects for routine evaluation should be immersion of the tissue or suspended cells in 4% paraformaldehyde, optimally only for a brief period of fixative exposure (<24 h), with subsequent resuspension in a flow cytometric (e.g. FACs) or other buffered solution. Regardless of the overall processing/storage decisions, all specimens (including controls) should be processed in the same fashion and prior to fixation or freezing, performed at 4 °C. Importantly, optimization and validation should be performed not only for the selected cell processing/storage but also for each marker of interest since fixation and freezing can impact the expression of various markers.

6.3.6 Antibodies

An understanding of the system to be evaluated, species-specific differences in cell populations of interest, and availability/quality of antibodies are critical factors that can impact data acquisition, analysis and interpretation. Many more species-specific antibodies are available now than ever before, so major cell classes (B, T and NK cells, myeloid and granulocytic cell populations and even subpopulations such as T_{regs}) often can be evaluated. For early discovery and research studies involving mice, numerous mouse-specific antibodies are available. However, although cross-species reactivity can be found with some antibodies, the lack of species-specific antibodies is a common limitation on flow cytometric assessment for many markers of interest in the rat, dog and non-human primates, species most commonly used for toxicity/safety assessment. Additionally, within non-human primates, there are differences in antibody cross-reactivity even between different species (e.g., rhesus macaques [*Macaca mulatta*] versus cynomologus macaques [*Macaca fascicularis*]) (Autissier et al. 2010; Njemini et al. 2014). Selection of reagent panels can be particularly complex depending on the instrumentation, available antibodies, emission/excitation characteristics and spectral overlap of the fluorophores and any autofluorescent molecules (i.e., "background"), antigen-antibody saturation, optimal signal-to-noise ratios, antigen density in target cell populations, antibody specificity, steric hindrance, and epitope number and complexity (Hulspas et al. 2009; Maecker et al. 2004; Schwartz et al. 2004; Tanqri et al. 2013). Significant planning and the utilization of appropriate reagent and cellular controls are critical when choosing and designing antibody panels employed for flow cytometric analyses of immune cells.

6.3.7 Data Acquisition, Compensation, Controls and Thresholds

In flow cytometry, many variables must be considered in preparing to harvest and process samples. Due to the often broad emission spectra of some fluorochromes or dyes, one fluorochrome may contribute a large range of signal which is detected by more than one PMT or "spill over" into more than one channel. Compensation is the process of removing the "spill-over" signals by mathematical means to eliminate spectral overlap, thereby providing a clearer signal (Raveche et al. 2011; Roederer 2001, 2002). In modern practice, manual (analog) compensation (which typically has to be performed prior to analyzing samples) has been replaced in large part by digital (automated) compensation, which can be undertaken either pre- or post-sample collection. Compensation may be done by instrument-based and bead-based means (http://www.bdbiosciences.com/documents/Compensation_Multicolor_TechBulletin.pdf). Instrument-based compensation has traditionally been performed, particularly in an academic setting, but increasingly, commercially-available compensation beads are being employed. Compensation beads provide distinct positive and negative stained populations of known fluorescent intensity and are often ideal for dimly expressed antigens and/or antibodies that have low affinity. With validation requirements and high throughput often required in drug development, bead-based compensation is likely preferred.

A threshold (or "gate") for detection of a particular signal determines the wavelengths over which light emissions from a given reagent will be processed and analyzed. Such electronic gates can be set using known excitation and emission wavelengths for particular fluorophores, and in the presence of multiple labeled reagents can be employed to define specific subpopulations of immune cells—including the sorting of the cells into distinct fractions for further analysis. For example, a total population of collected blood cells can be evaluated by forward scatter and side scatter using a gate to ascertain whether or not they are CD3+, a marker of lymphocytes. From this gated CD3+ population, additional expression using other gates can be explored to identify whether or not these CD3+ cells are CD4+ or CD8+, thereby determining the relative percentages and ratio of helper T cells (CD3+CD4+) to cytotoxic T cells (CD3+CD8+). Figure 6.1 demonstrates an example of basic immunophenotyping utilizing various gates to discriminate different cell lineages in this manner.

Controls are samples that are used to help determine the degree of compensation, establish thresholds, and validate the quality of data, which together ensure that the instrument and assays are working effectively. A variety of possible controls can be included in a flow cytometric assay; examples of possible controls for immunophenotyping experiments include positive and negative controls for the cells/population of interest and for the antibody, isotype controls, isoclonic controls and fluorescence minus one (FMO) controls. Depending upon the rigor of the assay, the research question, the protocol and the preferences of the flow cytometrist, the number of controls may outnumber the actual number of samples analyzed. There is some debate regarding what controls are definitively necessary in any flow cytometric

analysis. Internal negative and positive controls can help validate instrumentation and confirm the relevance of the threshold levels used for detecting a particular marker of interest. Isotype controls are antibodies identical to the one used to bind to the marker of interest but with variable regions that lack specificity to the target marker. The purpose of isotype controls is to confirm the specificity of primary binding and rule out other cellular/protein interactions or non-specific Fc receptor binding as potential explanations for the detected signal. For example, an isotype control for rat anti-mouse CD4 IgG_1-FITC would be rat anti-mouse IgG_1-FITC. Although commonly employed, isotype controls are not considered necessary or even recommended to set thresholds for positive and negative samples. Internal cellular controls (e.g. negative cells) are considered to be equally valid for determining the lowest levels for which antibody binding will be considered negative, although this concept is the subject of debate (Tanqri et al. 2013). Isoclonic controls, which are a mixture of fluorochrome-conjugated antibodies and excess unlabeled antibodies, are less commonly employed as controls. Fluorescence minus one (FMO) controls are commonly used to interpret flow cytometry data (Feher et al. 2014). In an FMO control, all fluorochromes in a panel except the one that is being measured are included in a flow tube. Inclusion of all these reagents except the one of interest allows the technician to set detection levels for the specific marker of interest by controlling for the contribution and potential interactions of all other antibodies within that single sample tube. Controls such as these not only increase the rigor of the qualitative assessment typically ascribed to flow cytometry but also facilitate the increasing need for quantification of specific cell lineages that is necessary in the clinical setting of drug development.

6.3.8 Data Analysis and Reporting

Data analysis and reporting are key components of obtaining useful flow cytometry data (Pedreira et al. 2013). Several software packages (e.g. FlowJo, FCS Express, Flow2Go, FIND, Phaedra, etc.) are commercially or freely available for analyzing data although such programs often require considerable training and experience in flow cytometry to optimize the assessment. (Dabdoub et al. 2011; Pedreira et al. 2013; Robinson et al. 2012; Cornelissen et al. 2012). Figure 6.2 outlines some of the potential gating strategies that can be employed to evaluate routine, enhanced and functional immunophenotyping. Selection of an experienced individual to interpret and report flow cytometry data is critically important given the potential subjective nature of setting positive and negative thresholds. In addition, flow cytometric data ultimately will have to be evaluated within the larger context of any study, which again requires considerable expertise in flow cytometry and fundamental immune system biology and pathology. Ideally, an individual or group of individuals experienced with interpreting flow cytometric data in the context of other immunopathological and immunotoxicological data help provide a more robust and comprehensive picture of immune-based alterations and their potential relationship to test article

exposure during the course of drug development. As with all other aspects of evaluating safety and mechanisms of action of potential therapeutics, effective communication between toxicologists, pathologists, immunologists and others professionals is necessary to develop an integrated picture of the mechanism of action, toxicity and efficacy of any potential therapeutic.

Reporting requirements for immunotoxicity data are provided in other chapters of this book, and are not described in detail here. Briefly, however, immunophenotyping reports should include the common elements that should be present in any regulatory report. The nature of the reagents and controls (including the vendors and lot numbers) as well as details of the sample acquisition and processing protocols should be stated clearly. Specific details with respect to fixation, wash and staining (e.g., buffers, incubation times and temperatures) should be given. Text explanations of the major findings should be accompanied by representative figures that show the typical labeling pattern for control and test article-treated samples for all markers; where necessary for clarity and to support the interpretation, similar images should be shown for control specimens as well. The visual nature of graph-based flow cytometric data can justify the inclusion of histograms for all samples in an appendix of the report.

6.3.9 Validation

Validation is a critical component of any assay employed during the drug development process. Numerous resources are available which detail more of the processes and protocols required for effective validation and standardization procedures needed in drug development (Barnett et al. 2013; Davis et al. 2013; Davis et al. 2011; Ezzelle et al. 2008; Ferbas and Schroeder 2011; Green et al. 2011; Hill et al. 2011; Lamoreaux et al. 2006; O'Hara et al. 2011; Owens et al. 2000; Tanqri et al. 2013; Wood et al. 2013). The International Council for Standardization of Haematology (ICSH) and International Clinical Cytometry Society (ICCS) have formulated guidelines on aspects of validating cell-based fluorescence assays that address preanalytical and post-analytical considerations, as well as assay performance criteria. Various experts have put forth recommendations for flow cytometric instrumentation and assays applicable to drug development (Barnett et al. 2013; Davis et al. 2013; Davis et al. 2011; Ezzelle et al. 2008; Ferbas and Schroeder 2011; Green et al. 2011; Hill et al. 2011; Lamoreaux et al. 2006; O'Hara et al. 2011; Owens et al. 2000; Tanqri et al. 2013; Wood et al. 2013). General considerations for validating flow cytometry assays to be used in nonclinical immunotoxicology, GLP-compliant assays and assays for clinical monitoring are comparable to validating other assay kinds in drug development. These include such aspects as proficiency testing, sample handling, sample preparation, accuracy, specificity, sensitivity and precision within the service itself, but also reagent and method validation, validation of an instrument's setup, qualification, resolution, monitoring of the instrument's performance in parameters, data analysis and interpretation and quality control in all aspects (36).

6.4 Integration of Flow Cytometry with Other Investigative Techniques in Drug Development

Flow cytometry is a powerful tool that has broad applications in drug discovery and development. The application of 4 to 12+ -color flow cytometry can be useful to identify very specific subsets of cells that cannot be readily identified by routine histopathology, enhanced immunohistopathology, clinical pathology endpoints (e.g., cell differential counts), or leukocyte-specific immunohistochemistry (IHC). However, given that flow cytometry samples must be available in a single cell suspension for flow cytometric interrogation, the orientation and cellular locations of measured cells within a three-dimensional immune tissue cannot be ascertained with flow cytometry. Therefore, flow cytometry data should be considered in conjunction with such structure-based assessments as histopathology, enhanced immunohistopathology, and/or IHC. Indeed, flow cytometric data is most powerful when evaluated within the context of other immunopathologic methods (Lappin and Black 2003).

6.5 Conclusions

Responses of the immune system to NTEs, chemicals and toxins are increasingly being recognized as representing a spectrum. Simple determination of lymphopenia by hematologic evaluation or bone marrow toxicity (myelotoxicity) by morphological analysis is no longer considered to be sufficient for describing immune responses. The reason for this shift is that such techniques often fail to identify subtle changes in immune responses, especially those involving modifications to small discrete subsets of immune cells or particular kinds of immune outcomes (e.g., immunostimulation, immunoregulation, hypersensitivity and allergy). Additionally, given the wide array of NTEs which either directly aim to modify immune responses or that do so inadvertently, modern risk assessment for possible immune system effects requires increasing sophistication in assaying and interpreting the responses of the immune system. Flow cytometry represents a vital means for addressing such subtle changes in specific cell populations and functions.

Appropriately designed studies and data sets provided by flow cytometry during early drug discovery can help identify mechanisms of action, receptor-binding patterns and the potential for immunosuppression or immunotoxicity early in the pipeline, thereby permitting researchers to optimize lead candidate selection to avoid potential immunological complications. During toxicology studies, effective data analysis by an individual or groups experienced in immunopathology and other complementary areas (e.g., toxicology, immune-based functional assays) provides a more comprehensive view of immune system alterations. Finally, in clinical monitoring for human trials, flow cytometric data sets that directly provide immunogenicity or biomarker data can help evaluate efficacy early on and monitor for any

potential adverse events or side effects during later phases. In this fashion, flow cytometry represents not only an important platform for assembling nonclinical data sets for immunotoxicity but also a crucial tool for translating animal data to predict possible human risk.

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Chapter 7
Pathology Evaluation for Detection of Immunomodulation

George A. Parker and Paul W. Snyder

Abstract Detection of immune system effects associated with xenobiotic administration in nonclinical toxicology studies is partially dependent on pathology-related parameters, including hematology, clinical chemistry, and urinalysis assays, gross necropsy observations, terminal body weight and organ weight analysis, and histopathologic examination. Findings in these parameters may be influenced by numerous factors, including the species, sex, age, and nutritional status of the animals, as well as presence of any spontaneous or intercurrent disease processes. Findings identified during standard pathology evaluations may be confirmed or enhanced by specialized procedures such as immunophenotyping by flow cytometry, enhanced immunohistopathologic examination, functional assays, assays for cytokines and other signaling or effector molecules, and host resistance assays. Stress responses can impact the morphology and function of immune system organs, and commonly confound interpretation of suspected xenobiotic-associated changes in immune system parameters. Studies conducted in juvenile animals, or those involving biologics or immunomodulatory experimental procedures or vehicles present special problems in interpretation.

Keywords Histopathology • Histology • Immunohistopathology • Histomorphology • Microanatomy • Juvenile studies • Infusion studies • Adverse/non-adverse • Concurrent disease

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Abbreviations

α1-CPI	α1-cysteine proteinase inhibitor
α2M	α2-macroglobulin
AAT	α1-antitrypsin
ACTH	Adrenocorticotrophic hormone
ACTH	α1-antichymotrypsin
ADA	Anti-drug antibody
APC	Antigen-presenting cell
ARR	Antigen-retaining reticulum
BALT	Bronchus-associated lymphoid tissue
CAR	Cilia-associated respiratory
CD	Clusters of differentiation
CLR	C-type lectin receptor
CRD	Chronic respiratory disease
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
DART	Developmental & reproductive toxicology
FC	Fraction crystallizable
FDA	U.S. Food & Drug Administration
FDC	Follicular dendritic cell
FOB	Functional observational battery
GC	Germinal center
H&E	Hematoxylin and eosin histologic stain
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MAPK	Mitogen-activated protein kinase
NALT	Nasopharynx-associated lymphoid tissue
NBF	Neutral buffered formalin
NK	Natural killer
NLR	NOD-like receptor
NOAEL	No observed adverse effect level
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PDG	Polyethylene glycol
RLR	RIG-1-like receptor
SAA	Serum amyloid A
SRPC	Scientific & Regulatory Policy Committee of the Society of Toxicologic Pathologists
STP	Society of Toxicologic Pathologists
TDAR	T-dependent antigen response
TLR	Toll-like receptor

TNF	Tumor necrosis factor
V(D)J	Variable diversity joining gene segments
WBC	White blood cells

7.1 Introduction

The pathology component of typical nonclinical toxicology protocols is designed to provide a broad-based mechanism for detection of test article-related alterations in structure or function of major organs and tissues, with no particular emphasis on individual organs or tissue. Standard protocols may be modified based on intended route of administration, specific issues or findings that have occurred in previous studies of the same or similar test articles, or other factors. The standard pathology component of routine non-clinical toxicology studies is designed to be a screening tool rather than a definitive investigation of any organ system including the immune system.

Standard pathology evaluations are considered adequate for detection of test article-related alterations in many organ systems, but have been found inadequate for detection of chemically mediated changes in some organ systems. Additional specialized testing procedures are frequently necessary for detection or confirmation of immunomodulation, as is the case for a number of other organs and functional systems. Specialized procedures such as enhanced immunohistopathology, immunohistochemistry, immunophenotyping, and cytokine analysis should be used to address a specific finding identified in routine studies. Confirmation of suspected immunomodulation requires additional specialized methods such as cytokine analysis or T-dependent antigen response (TDAR), NK cell and CTL activity, and host resistance assays.

Alterations in the immune system are generally characterized as suppressive or enhancing responses. Immunosuppression most commonly results in an increased susceptibility to infections while enhancing responses are manifested as hypersensitivity reactions. Unfortunately, the pathology component of routine non-clinical toxicology studies is not specifically designed to adequately identify all of these alterations. While it is possible that standard pathology evaluations may detect the morphologic alterations associated with immunosuppression, those evaluations are unlikely to detect misdirected or enhanced immune responses. Testing methods other than those in standard pathology evaluations will almost certainly be required for detection of these latter types of immunotoxicants.

Pathology evaluations for purposes of this discussion are subdivided into: (1) necropsy examination; (2) organ weight evaluation; (3) clinical pathology; (4) histopathology; and (5) special procedures. Table 7.1 presents typical clinical pathology evaluations, Table 7.2 presents a typical list of tissues that are preserved for histopathological evaluation, and Table 7.3 presents organ weight analysis for a typical non-clinical toxicology study in rats.

Following is a brief discussion of these individual evaluations as they relate to the detection of immune system toxicities.

Table 7.1 Clinical pathology evaluation for typical non-clinical toxicology study

Hematology and coagulation	
Total leukocyte count	Red cell distribution width
Erythrocyte count	Hemoglobin distribution width
Hemoglobin	Differential absolute leukocyte count -
Hematocrit	- Neutrophil
Mean corpuscular volume	- Lymphocyte
Mean corpuscular hemoglobin	- Monocyte
Mean corpuscular hemoglobin concentration	- Eosinophil
Platelet count	- Basophil
Mean Platelet volume	- Large unstained cell
Prothrombin time	Reticulocyte count
Activated partial thromboplastin time	Percent
	Absolute
Serum chemistry	
Albumin	Gamma glutamyltransferase
Total protein	Glucose
Globulin [calculated]	Total cholesterol
Albumin/globulin ratio [calculated]	Calcium
Total bilirubin	Chloride
Urea nitrogen	Phosphorus
Creatinine	Potassium
Alkaline phosphatase	Sodium
Alanine aminotransferase	Triglycerides
Aspartate aminotransferase	Sorbitol dehydrogenase
	Appearance[a]
Urinalysis	
Specific gravity	Glucose
pH	Ketones
Urobilinogen	Bilirubin
Total volume	Occult blood
Color	Leukocytes
Clarity	Nitrites
Protein	Microscopy of sediment

[a] Includes the degree of hemolysis, icterus, and lipemia

7.2 Necropsy and Histotechnology

Animals that die during non-clinical toxicology studies and those sacrificed at the termination of dosing are subjected to a thorough gross necropsy examination. Interim phase as well as recovery phase sacrifices are commonly employed to further characterize test article effects.

Some alterations are more amenable to detection by gross inspection. Among these are discolorations that may not persist through histologic processing, distention of organs that may revert to normal upon fixation, and accumulation of fluids or gases that may be removed during histologic processing. Many xenobiotic-related morphologic alterations are commonly detected by histopathologic examination and organ weight analysis (Sellers et al. 2007a; Crissman et al. 2004). Careful

Table 7.2 Tissues preserved[a] for histopathological evaluation from typical non-clinical toxicology study

Adrenal glands (2)	Nasal cavity (not including olfactory bulbs)[d]
Aorta	Ovaries (2) with oviducts
Bone with marrow	Pancreas
Femur with joint	Peripheral nerve (sciatic)
Sternum	Peyer's patches
Bone marrow smear (from femur)[a]	Pharynx[d]
Brain	Pituitary
Cervix	Prostate
Epididymides (2)[b]	Salivary glands [mandibular, parotid, sublinguinal (2)]
Eyes with optic nerves (2)[c]	Seminal vesicles (2)
Gastrointestinal tract	Skeletal muscle (rectus femoris)
Esophagus	Skin with mammary gland
Stomach	Spinal cord (cervical, thoracic, lumbar)
Duodenum	Spleen
Jejunum	Testes (2)[b]
Ileum	Thymus
Cecum	Thyroid with parathyroids (2)
Colon	Tongue
Rectum	Trachea
Heart	Urinary bladder
Kidneys (2)	Uterus
Larynx[d]	Vagina
Liver (sections of 2 lobes)	Gross lesions
Lungs (including bronchi, fixed by inflation with fixative)	
Lymph nodes	
Mandibular (2)	
Mesenteric	

[a] Standard fixative is 10% neutral buffered formalin (NBF)
[b] Fixed in modified Davidson's solution
[c] Fixed in Davidson's solution
[d] Collected and held in formalin (not routinely sectioned).

Table 7.3 Organs for weight analysis in typical non-clinical toxicology study

Adrenal glands	Prostate with seminal vesicles
Brain	Pituitary
Epididymides	Spleen
Heart	Testes
Kidneys	Thymus
Liver	Thyroid with parathyroids*
Ovaries with oviducts	Uterus

Paired organs are weighed together. Designated (*) organs are weighed after fixation. Organ to final body weight and organ to brain weight ratios are calculated

examination during necropsy, meticulous recording of observations, group-related tabulation of observations and methodical tracking of gross lesions through histologic processing and histopathologic examination are required if macroscopic and morphologic alterations are to serve as a meaningful basis for interpretation.

7.2.1 Fixation of Tissue Specimens for Routine Histopathological Evaluation

Tissue specimens for histologic processing are commonly preserved ('fixed') in 10% neutral buffered formalin (NBF). Buffering of the fixative solution is accomplished by the addition of various salts, commonly monobasic and dibasic sodium phosphate. Without buffering and pH adjustment, formalin fixation may result in the formation of acid hematin that can interfere with detailed histopathological evaluation (Pizzolato 1976).

It should be noted that pure formaldehyde is a gas. The commonly used 10% NBF fixative solution is a 1:9 dilution of formaldehyde solution, the latter being only 37–39% formaldehyde, thus the final 10% NBF fixative solution is only 3.7–3.9% formaldehyde. Misunderstandings regarding the terminology used for this common fixative solution can result in serious deficiencies in tissue preservation and fixation. Commercially available formaldehyde solution commonly contains a variable amount of methanol. In addition, formaldehyde polymerizes upon prolonged storage to form a precipitate, which reduces the amount of formaldehyde that is available for tissue fixation. As a result of polymerization and methanol conversion, the actual formaldehyde content of commercial formaldehyde solution may be difficult to ascertain. In situations where precise tissue fixation is important, it is best to use freshly prepared solutions of paraformaldehyde for fixation.

Prolonged immersion fixation in aldehyde fixatives such as 10% NBF results in aldehyde-mediated cross-linking of peptides, eventually resulting in an occlusive matrix that can "mask" the target protein, thus reducing or preventing binding of the primary antibodies that are the critical first step in immunohistochemical (IHC) staining. While it is sometimes possible to reverse the cross-linking effects, these 'de-masking' procedures introduce additional variables into the experimental procedure and should be avoided when possible. A common method of limiting aldehyde-mediated peptide cross-linking involves limiting the immersion fixation of tissues in formalin to $\leq$48 h, followed by transfer of tissue specimens to 70% ethanol solution while awaiting routine histologic processing. When using this 'IHC-optimized' fixation regimen, histology personnel should substitute 70% ethanol for the 10% NBF that commonly occupies the first processing station on automated tissue processors.

Additional tissue fixatives and fixation procedures for immunohistochemical staining have been proposed (Arnold et al. 1996; Beckstead 1994; Braber et al. 2010; Ramos-Vara 2005; Yan et al. 2010; Mori et al. 2015).

7.2.2 Histotechnique Considerations for Immune System Organs

The histopathologic evaluation in non-clinical toxicology studies typically includes H&E-stained sections of bone marrow, thymus, spleen, and lymph nodes that drain routes of exposure (Haley et al. 2005; Kuper et al. 2000; Schuurman et al. 1994).

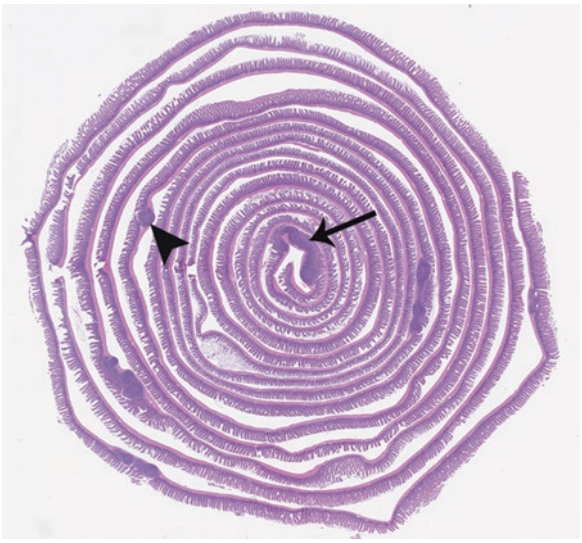

Fig. 7.1 Swiss roll preparations of the small intestine of rats allows microscopic examination of the entire length of the small intestine, including Peyer's patches (*arrow*) and individual lymphoid follicles (*arrowhead*). In this preparation the ileum is placed at the center of the roll, and the duodenum is at the outside margin of the roll. Note that Peyer's patches are distributed throughout the small intestine of rats. There are no definitive histologic landmarks between the regions of the small intestine, though the different regions have histologic features that are characteristic of that particular region. If it is important to localize test article effects to a specific region of the small intestine, the study protocol must provide specific guidance on how those regions are defined. This is commonly accomplished by specifying linear distances from some clearly identifiable point, e.g. the point of entry of the common bile duct into the anterior duodenum or the ileocecal junction at the distal end of the small intestine. H&E stain, 0.47× objective magnification

Some laboratories require examination of a 'non-enteric' lymph node such as the axillary or popliteal lymph node, and examination of Peyer's patches in the small intestine is commonly required by modern protocols. Examination of Peyer's patches is commonly accomplished by preparing histologic sections of one or two grossly visible Peyer's patches but, in situations where more extensive evaluation is required, the 'Swiss-roll' technique may be employed to permit examination of the entire small intestine (Fig. 7.1) (Moolenbeek and Ruitenberg 1981). Bronchus-associated lymphoid tissue (BALT) is commonly included in the lung sections in species that have constitutive expression of BALT. If study protocols require examination of nasal cavity, e.g., in inhalation studies, then nasopharynx-associated lymphoid tissue (NALT) is commonly present for evaluation in the most caudal nasal cavity section. Instructions for gross tissue trimming of rodent specimens and examples of resultant histologic sections have been published (Kittel et al. 2004; Morawietz et al. 2004; Ruehl-Fehlert et al. 2003), and are available online at www.goRENI.org.

Histologic sections from non-clinical toxicology studies are commonly 5–6 micrometers in thickness, and are stained with hematoxylin and eosin (H&E). Such sections are adequate for detection of xenobiotic-associated histologic alterations in most tissues, but

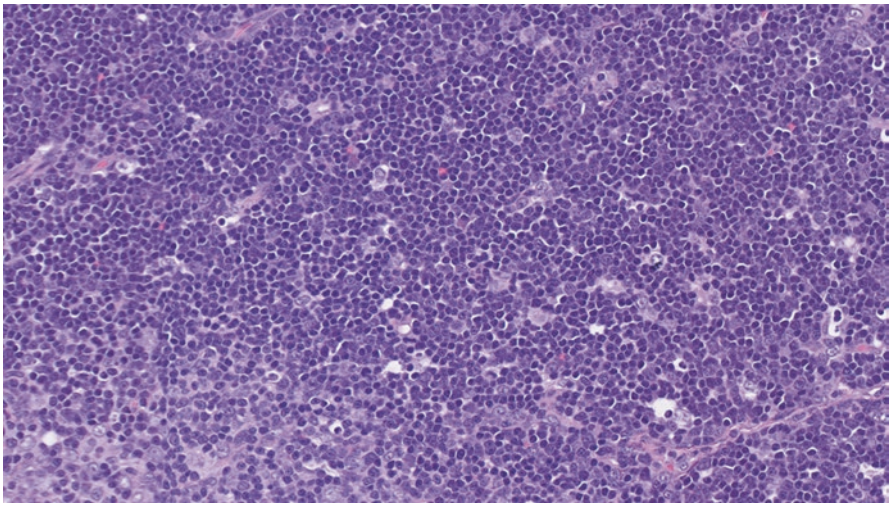

Fig. 7.2 The thymic cortex of a rat sectioned at 3 μm thickness allows features of individual cells to be distinguished. H&E stain, 40× objective magnification

the sections may be too thick for critical evaluation of lymphoid tissues. Thinner sections (2–3 micrometers) may be necessary to delineate some changes, particularly in tissues such as the thymus that have dense lymphocyte populations. (Fig. 7.2).

Histologic sections of sternum and/or femur with in situ bone marrow are commonly examined as part of the routine histopathological evaluation. Many laboratories also prepare bone marrow smears from the contralateral femur, and hold smears for use in cytologic evaluation if indicated by results of the histologic evaluation. Both histologic and cytologic evaluation have value in the overall interpretation of bone marrow. Histologic evaluation of the marrow *in situ* in the sternum and/or femur allows the pathologist to evaluate overall marrow cellularity and subjectively estimate the adequacy of specific cell populations, particularly megakaryocyte numbers. If properly preserved, sectioned and stained, histologic sections can be used to estimate myeloid: erythroid (ME) ratios of bone marrow. Cytologic evaluation allows definitive identification of cell types, including the developmental stages of the various cell lines. Cytologic evaluation is preferable when detailed ME ratios are needed. The quality of histologic sections of bone marrow is greatly influenced by the decalcification procedure, which involves a balance between preservation of marrow cell features versus adequate removal of mineral to allow histologic sectioning. Excessive decalcification, while simplifying the histologic sectioning step, may reduce the integrity of the marrow cell components to the point that histologic evaluation of the bone marrow is inadequate. Formic acid-based decalcification procedures are widely used in non-clinical toxicology studies and are satisfactory in preserving marrow cell features if careful attention is given to the decalcification time. A specialized formic acid-based decalcification procedure has been reported (Kristensen 1948), and is known to give excellent results in animals on non-clinical toxicology studies.

7.3 Organ Weight Evaluation

The lymphoid organ weight organ abnormalities most commonly identified in routine non-clinical toxicology studies involve absolute or relative weights that are lower than concurrent control values. Although significantly lower lymphoid organ weights can be associated with immunosuppression, organ weight changes are not reliable predictors of enhanced immune responses. Interpretation of lower immune system organ weights in xenobiotic-treated animals is complicated by secondary stress responses, which can also result in lower immune system organ weights, particularly the thymus, in some species. Organ weight interpretation is further complicated by xenobiotic-related decreases in body weight, which may or may not be due to decreased feed consumption. Individual organs have variable sensitivities to body weight decreases. The weight of some organs change synchronously with body weight decreases while the weights of other organs are conserved despite decreases in body weight. Finally, the weight of other organs is influenced to an intermediate degree by decreases in body weight. Many non-clinical toxicology studies are conducted on immature, actively growing animals, thus organ weights are expected to increase during the course of the study. Changes in the weight of individual organs may present a different pattern in animals that actually lose weight, as opposed to animals that fail to gain weight during the course of the study. Because brain weight tends to be conserved despite a decrease in body weight or failure to gain body weight, brain weight is commonly used as a benchmark reference relative weight for comparison to other organ weights (Michael et al. 2007; Bailey et al. 2004; Sellers et al. 2007b). If the true significance of organ weight alterations is to be accurately interpreted, organ weight changes should be evaluated as absolute organ weights, relative to body weight and relative to brain weight (Schafer 2007; Scharer 1977; Schwartz et al. 1973). Careful attention should be given to feed consumption or nursing data as well as any in-life, gross necropsy, clinical pathology, serologic, or histopathologic evidence of spontaneous disease or other factors that could indicate intercurrent disease or a stress response in individual animals.

7.4 Histopathology

As a preliminary step in the histopathologic evaluation, it is sometimes helpful to directly compare histologic sections by sub-gross inspection prior to microscopic examination. This may reveal general trends in organ size that are less apparent upon microscopic examination. Any observations relative to the sub-gross examination of histologic sections should be compared to organ weight data.

Microscopic changes associated with altered immune responsiveness may consist of (a) morphologic alterations in the primary or secondary lymphoid tissues and/or (b) secondary evidence of altered immune system function with resultant changes in non-lymphoid organs. Normal and abnormal morphologic features of the major

immune system organs of rodents are reviewed in a monograph (Cesta 2006a, b; Elmore 2006; Kuper 2006; Maronpot 2006; Pearse 2006a, b; Suttie 2006; Travlos 2006a, b; Willard-Mack 2006), and are presented in Volume 2, Chaps. 1–4.

Information presented herein may serve as a general guideline for histologic evaluation of immune system organs in strains of rat other than the Sprague-Dawley, but detailed study of immune system organs from the various rat strains would be necessary to make specific comparisons. Several strain-related differences in immune system structure and responses have been reported (Bunn et al. 2001a; b; Bruder et al. 1999), and undoubtedly additional differences remain to be identified.

7.4.1 Morphological Alterations in Organs of the Immune System

The primary indicators of immunotoxicity in general toxicology studies include the total white blood cell count (WBC) and leukocyte differential counts in the hemogram, total protein, albumin, globulin and liver enzyme levels in the clinical chemistry analysis, body and lymphoid organ (typically thymus and spleen) weights, and histopathologic evaluation of routine H&E-stained sections of thymus, bone marrow, spleen, lymph nodes and Peyer's patches of the small intestine (Hinton 2000). The U. S. FDA "Redbook II" immunotoxicity testing guidelines recommend expanded level I histopathologic evaluation, which is defined as "any procedural or mechanical modification in tissue processing, staining, or visualization that improves upon either precision or sensitivity, resulting in greater descriptive or diagnostic capability in pathologic evaluation of the lymphoid (or other) organs"(Hinton 2000). The expanded level I histopathologic evaluation requires detailed histopathologic observations that give some indication of affected compartments in immune system organs, as opposed to generic observations of effects on entire immune system organs (Schuurman et al. 1994; Kuper et al. 1995).

Decreases in the population of lymphocytes in the thymic cortex is a common manifestation of immunosuppression in animals of the age used in short-term (1–13 weeks in duration) non-clinical toxicology studies. A lower ratio of cortical to medullary areas is sometimes recorded as lymphoid atrophy or lymphoid depletion. Lymphoid atrophy/depletion may result from increased rate of death of cortical lymphocytes, or decreased proliferation rate of cortical lymphocytes. The high proliferation rate of cortical thymic lymphocytes (Fig. 7.3) makes this cell population uniquely sensitive to disruptions in proliferation rates, resulting in a rapid reduction in the overall cellularity of the thymic cortex. Conversely, the high proliferative rate of cortical thymic lymphocytes allows for rapid reversal once the stimulus is removed. Lower thymic cortical cellularity related to test article administration must be distinguished from normal involution of the thymus, which is histologically similar. Review of the age of individual animals may be helpful in making the latter distinction, though experience suggests there is great variation in the age of onset and rate of progression of age-related changes in the thymus of the common laboratory

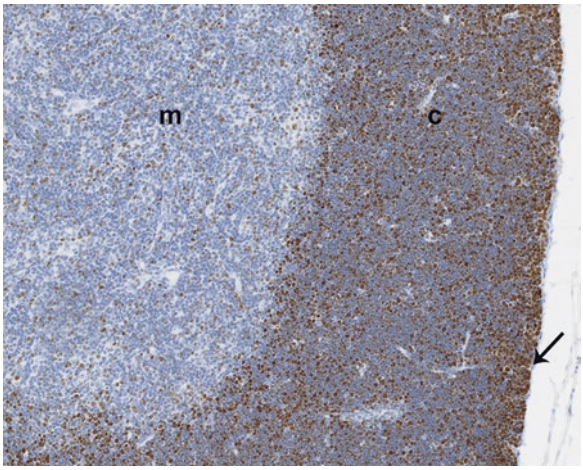

Fig. 7.3 An immunohistochemical stain for a cellular proliferation marker shows intense proliferative activity in the thymic cortex (*c*), with less proliferation in the medulla (*m*). Note the intense band of cellular proliferation immediately beneath the capsule (*arrow*). Ki67 immunohistochemical stain with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 15× objective magnification

animals. There is a particularly high level of variability in thymus weights in cynomolgus macaques, to the point it is difficult to utilize thymic weight data in interpretation of potential immunomodulation in that species (Snyder et al. 2016).

Depending on the interval between the insult and tissue sampling, histologic evidence of active degeneration or apoptosis of thymic cortical lymphocytes may not be evident. While these morphologically different histological changes probably progress to a final stage that is identifiable as lymphoid depletion, thus represent different stages in the same pathologic process, the active degenerative/apoptotic lesions should be identified by some distinctive term that allows them to be distinguished from the inactive terminal changes. This characterization of histologic changes in immune system organs is also helpful for subsequent study designs to address immune system alterations.

A majority (>95%) of immature thymic cortical lymphocytes fail to survive as a result of negative and positive selection processes. Histologic evidence of lymphocyte apoptosis in the thymus is characterized by fragmented or shrunken lymphocytes, sometimes contained with macrophages (‘tingible body macrophages’). Distinguishing this background level of cell death from test article-related changes can be challenging when the test article effects are subtle. Application of rigorous diagnostic criteria, including severity scoring criteria, is important in making these distinctions. As discussed below, digital image analysis performed on sections subjected to immunohistochemical staining for apoptosis markers may provide a more definitive indication of the level of apoptosis in the thymus.

Immunotoxicity may result in subtle morphological alterations in the immune system that are not readily classified by standard histopathologic evaluation, which is designed to detect and assign subjective severity scores to ‘lesions’ (Kuper et al.

1995, 2000; 2013; Hinton 2000). Immunotoxicity-associated morphologic alterations may fall within the broad range of 'histologically normal', thus would not be detected by standard histopathology procedures. Guidelines for enhanced histopathologic evaluations of the immune system organs have been proposed in an attempt to address some of the deficiencies of routine histopathology (Haley et al. 2005). The enhanced histopathologic evaluation consists of subdividing immune system organs into readily identifiable morphologic compartments and applying numerical severity scores to various morphologic attributes of the compartments, regardless of whether those attributes are judged to be 'normal' or 'abnormal' (Elmore 2007a, b, c, d, e, f). Details of enhanced histopathologic evaluation are presented in Volume 1, Chap. 8.

The histopathologic evaluation of non-clinical toxicology studies performed in support of candidate drug development is somewhat different from the evaluation that is performed on studies of suspected immunomodulatory environmental or industrial hazards. The latter studies often involve very low level xenobiotic exposures that result in subtle histologic alterations in immune system organs. Such studies are ideally suited for enhanced immunohistopathology evaluation, which in many ways is the 'gold standard' for histopathological detection of immunomodulation. By contrast, non-clinical toxicology studies for pharmaceutical product development are typically designed with the objective of identifying toxicities and involve higher dose levels that result in more overt alterations in immune system organs. In addition, some therapeutics involve deliberate targeting of specific aspects of immune system function. While the fully implemented enhanced immunohistopathology evaluation may not be warranted in preclinical pharmaceutical development studies, application of the basic premises of that type of evaluation can add value to the overall histopathological evaluation of the studies. Including details of treatment-related effects on the various compartments of immune system organs can be helpful in (a) confirming that observed changes are expected based on the known pharmacological activity of the test article, (b) identifying unexpected off-target immunomodulation, (c) identifying secondary complications of expected or unexpected immunomodulation, or (d) distinguishing xenobiotic-related immunomodulation from nonspecific stress responses (Everds et al. 2013). When preclinical studies are conducted in juvenile animals, compartment-based evaluation of immune system organs may aid in distinguishing between xenobiotic-related immunomodulation and normal postnatal development of immune system organs (Parker et al. 2015). Table 7.4 contains an outline of compartment-based evaluation of immune system organs that is based on the principles of enhanced immunohistopathology. It is important for the pathologist to use only descriptive rather than interpretative terminology when using this approach for recording potential xenobiotic-related structural alterations in immune system organs (Haley et al. 2005). For example, a thymus with thin, hypocellular cortex would be recorded as 'thymus, cortex-reduced cellularity' rather than 'thymus, cortex-atrophy' or 'thymus, cortex- lymphoid depletion' because the latter terms suggest the pathogenesis of the histologic alteration. The goal is for histopathologic observations to be purely observational, with any interpretations identified as such in the narrative pathology report.

Table 7.4 Outline of compartment-based histologic observations for immunomodulation

Bone marrow (individual bones recorded separately)
• Overall cellularity
• Adipocyte population
Thymus
• Cortex- size and cellularity
• Medulla- size and cellularity
• Corticomedullary distinction
• Tingible-body macrophage cellularity
Spleen
• Periarteriolar lymphoid sheaths- size and cellularity
• Follicles- size and cellularity
• Germinal centers- number and cellularity
• Marginal zone- cellularity
• Red pulp mononuclear cell population- cellularity
Lymph nodes (individual lymph nodes recorded separately)
• Overall cortex cellularity
• Follicles- size and cellularity
• Germinal centers- number and cellularity
• Paracortex- cellularity
• Medullary sinuses- cellularity
• Medullary cords- cellularity
• Plasmacytosis
Mucosa-associated lymphoid tissue (MALT) (Individual sites recorded separately)
• Overall cellularity
• Follicles- size and cellularity
• Germinal centers- number and cellularity

When dealing with developmental and reproductive toxicology (DART) studies, attention should be given to the placenta as a possible site of xenobiotic-mediated immunomodulation. Placentas are classified by shape (diffuse, discoid, zonary or cotelydonary) or degree of separation between maternal and fetal blood (epitheliochorial, syndesmochorial, endotheliochorial or hemochorial) (Bjorkman and Dantzer 1987; DeSesso 2011). Close approximation of maternal and fetal blood allows placental transfer of maternal antibodies to the fetus, while a greater separation between maternal and fetal blood necessitates colostral transfer of maternal antibodies after birth. The placenta of humans and rodents is hemochorial, which results in a very close approximation of fetal and maternal blood and resultant placental transfer of maternal antibodies to the fetus. The greater number of placental layers in species such as horses and cattle necessitates colostral transfer of maternal antibodies to the fetus. Failure of that early postnatal transfer can be life-threatening to the newborn. Placental involvement in feto-maternal immunity is addressed in greater detail in Volume 2, Chap. 11.

7.4.1.1 Germinal Center Reaction

The pathology evaluation in standard nonclinical toxicology studies typically includes histopathologic examination of primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs. A key microscopic feature of an active humoral immune response is formation of secondary lymphoid follicles characterized by distinct germinal centers (GCs) in the spleen and lymph nodes (Fig. 7.4a–f). Germinal centers are transient structures that form in secondary lymphoid organs in response to T lymphocyte-dependent antigens. The germinal centers are the primary sites for B lymphocyte activation, somatic hypermutation (Jacob et al. 1991; Berek et al. 1991) and affinity maturation resulting in clonal expansion of B lymphocyte populations (McHeyzer-Williams et al. 1993; Jacob et al. 1993), and generation of the B-cell memory response (Lane et al. 1994; Coico et al. 1983). The presence or absence of microscopically normal germinal centers in secondary lymphoid tissues is an important factor in the overall assessment of adaptive immune system integrity, therefore, a thorough understanding of the immunologic basis and morphologic correlates of the germinal center reaction is important in the detection and interpretation of immunomodulation in non-clinical toxicology studies.

The germinal center reaction involves at least four cellular components:

- Follicular dendritic cells that localize GC formation and serve as a long-term repository for antigen complexed with antibody and complement, which is required for maintaining continued B cell activation for a specific antigen (Klaus et al. 1980; Mandel et al. 1980; Tew et al. 1990; Schriever and Nadler 1992)
- Antigen-specific helper T cells (Kelsoe and Zheng 1993; Zheng et al. 1994; Fuller et al. 1993; Zheng et al. 1996)
- B cells (Jacob et al. 1991; Jacob and Kelsoe 1992; Berek et al. 1991; Liu et al. 1991; McHeyzer-Williams et al. 1993; Kroese et al. 1987)
- Macrophages that remove apoptotic lymphocytes.

Germinal center formation is dependent on localization of antigen in lymphoid follicles and trapping of antigen/antibody/complement complexes by follicular dendritic cells (FDCs). Cytoplasic processes of the FDCs establish an antigen-retaining reticulum (ARR) that can support the GC reaction (Nossal et al. 1968; Szakal and Hanna 1968; Szakal et al. 1983). The ARR is associated with the light zone of developing GCs in humans, (MacLennan 1994) (Szakal et al. 1983), but light and dark zones are rarely visible in lymphoid follicles of the animal species that are commonly used for non-clinical toxicology studies. FDCs, which are found only in primary and secondary lymphoid follicles, have multiple features which distinguish them from macrophages and other dendritic cells such as interdigitating dendritic cells of T cell areas or Langerhans cells of the skin. FDCs are non-bone marrow derived cells, non-phagocytic, lack lysosomes and Birbeck granules in their cytoplasm, and have the ability to retain intact antigens for a prolonged period of time. Antigen retention by follicular dendritic cells is dependent on the presence of complement (Dukor et al. 1970) (Pryjma and Humphrey 1975; Van den Berg et al. 1992) and antigen-specific antibodies (Tew et al. 1980), which suggest antigens on FDCs

are held as immune complexes. These complexes are visualized via electron microscopy as immune complex-coated bodies ('iccosomes') on FDC cell membranes and dendrites (Szakal et al. 1988). The retained antigen/antibody/complement complex constitutes an unprocessed reserve which may be maintained for an extended period of time (Nossal et al. 1968; Tew and Mandel 1979; Kosco 1991). These deposits of unprocessed antigen are necessary for the maintenance of serum antibody titers and establishment of memory B cells (Tew et al. 1980; Gray and Skarvall 1988).

In addition to intracellular signals mediated by the T cell receptor and B cell receptor, the germinal center reaction is dependent on a number of co-stimulatory signals for the antigen-driven proliferation of B cells and their differentiation into plasma cells. Two sets of interactions are of primary importance in generation of the germinal center reaction and T-B cell interactions in general: CD40 interaction with CD40L and CD80/86 (B7) interaction with CD28.

CD40 is expressed ubiquitously on antigen-presenting cells, including macrophages, follicular dendritic cells, interdigitating dendritic cells, B cells and some epithelial cells (Stamenkovic et al. 1989; Galy and Spits 1992; Galy et al. 1993), while CD40L is expressed primarily by activated (but not resting) $CD4^+$ T_H cells. If the CD40/CD40L interaction is blocked, B cells fail to proliferate and do not produce immunoglobulin in response to T_H cell signals (Armitage et al. 1992; Hollenbaugh et al. 1992; Lederman et al. 1992; Spriggs et al. 1992).

While the CD40/CD40L interaction allows B cells to respond to activated T_H cells, the reciprocal interaction between CD80/86 on B cells and CD28 on T cells allows the T_H cells to proliferate and produce cytokines in response to activated B cells. There is evidence that CD86 expression is necessary for the initiation of immune responses, while CD80 expression is more important for maintenance and amplification of the humoral immune response (Freeman et al. 1993). All normally functional T cells express CD28, which is the lower affinity ligand for CD80/86, while CTLA-4 is a higher affinity ligand for CD80/86 that is found only on activated T cells (Harper et al. 1991; Linsley et al. 1992).

Formation of germinal centers involves a complex series of interactions between various cell populations, and an intricate pattern of cell movements within the germinal centers. The initial step is interaction of antigen with IgM^+ IgD^+ naïve B cells in the primary follicles of secondary lymphoid organs such as lymph nodes. The antigen-specific B cells move to the periphery of the primary follicles to the junctional area between B cell-rich and T cell-rich areas, where they interact with follicular helper T (T_{FH}) cells. At this point, antigen-activated proliferating B cells of lymph nodes differentiate along one of three pathways. One subset migrates into medullary cords, where they differentiate into short-lived plasmablasts that produce relatively low-affinity antibody (Jacob and Kelsoe 1992). A second subset differentiates into germinal center-independent memory B cells (Taylor et al. 2012). A third subset, those with the highest affinity for specific antigen, enter the germinal center cycle and differentiate into long-lived plasma cells or germinal center-dependent memory B cells (Kurosaki et al. 2015).

This complex series of cellular interactions and movements results in the formation of antigen-specific plasma cells and memory B cells within 1 week of specific antigen exposure (Blink et al. 2005):

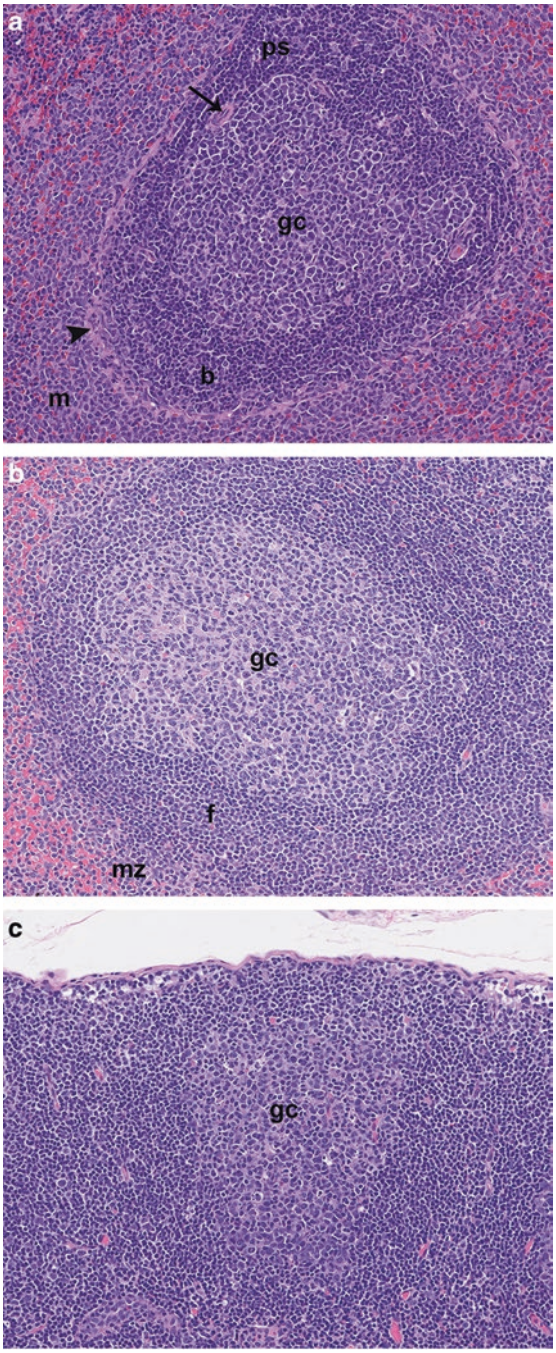

Fig. 7.4 (**a**) This secondary lymphoid follicle in the spleen of a rat demonstrates the major features of splenic lymphoid follicles. *m* = marginal zone, which contains marginal zone B cells, marginal zone macrophages, and metallophilic macrophages; *arrowheads* = marginal sinus; *b* = B cells of follicle; *arrow* = arteriole; *ps* = periarteriolar sheath; and *gc* = germinal center. Marginal zones and marginal sinuses are particularly prominent in the spleen of rats as compared to other species. Note that B cells of the follicle are not distinguishable from T cells of the periarteriolar

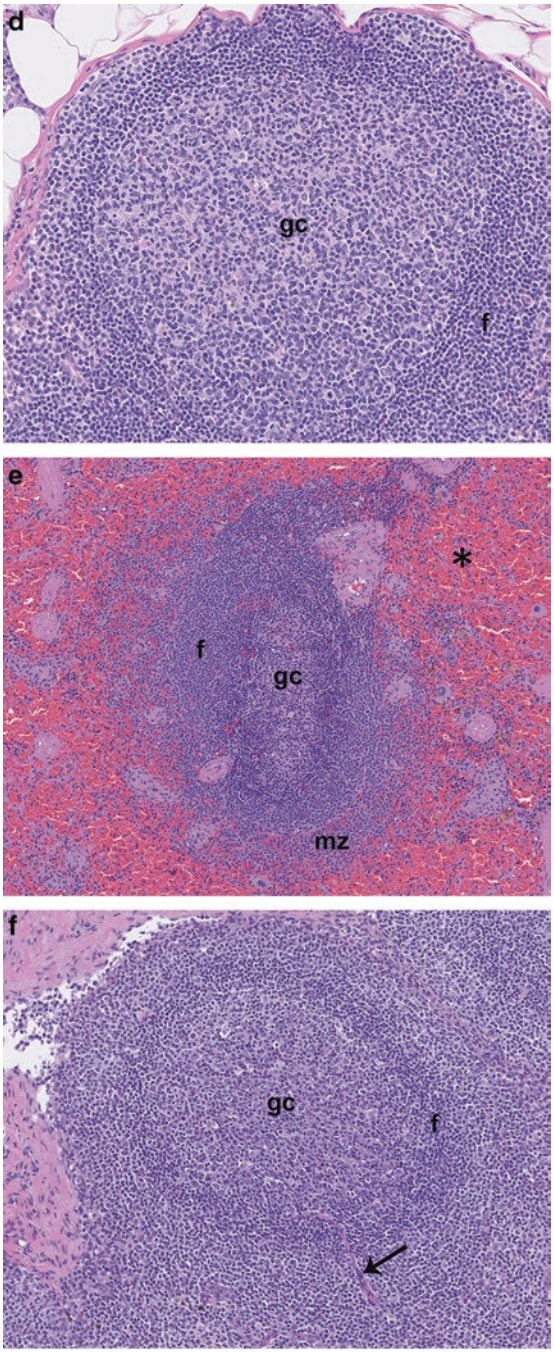

Fig. 7.4 (continued) lymphoid sheath except by location relative to the arteriole. Histologic sections represent a two-dimensional presentation of a three-dimensional structure, therefore all components and relationships will not be apparent in each follicle in a histologic section. H&E stain, 20× objective magnification. (**b**) This secondary follicle in the spleen of a cynomolgus macaque has a dense population of follicular lymphocytes (*f*) surrounding a central germinal center (*gc*). A marginal zone (*mz*) is present around the outer perimeter of the follicle, but is not as distinct as the

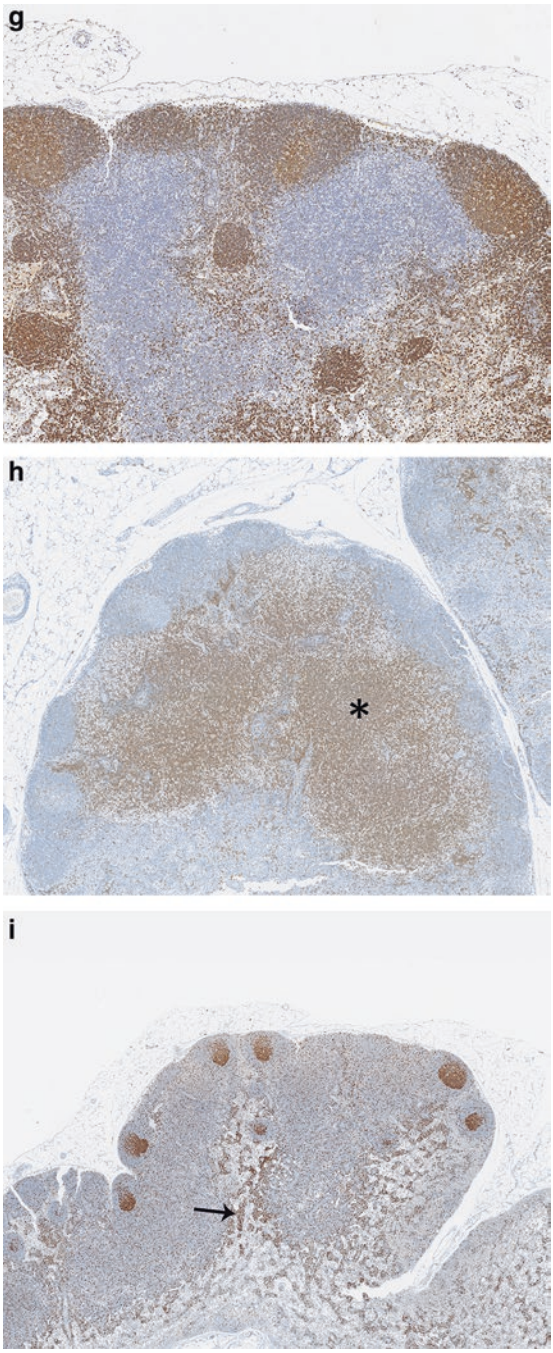

Fig. 7.4 (continued) marginal zones seen in rats. H&E stain, 20× objective magnification. (**c**) The mandibular lymph node of a Sprague-Dawley rat has a follicle that is denoted by the presence of a germinal center (*gc*), but the surrounding B cell population of the follicle blends imperceptibly into the diffuse B cell population of the lymph node cortex. H&E stain, 20× objective magnification. (**d**) The mandibular lymph node of a cynomolgus macaque has a secondary lymphoid follicle that consists of a central germinal center (*gc*) surrounded by a zone of follicular B cells (*f*). H&E stain,

- Day 1—Activated B cells and T cells migrate to the interfollicular zone and initiate the differentiation processes.
- Day 2—B cells are fully activated and T cells have acquired the characteristic T_{FH} phenotype.
- Day 3—T_{FH} cells migrate from the interfollicular region into the follicle.
- Day 4—B cells migrate from the interfollicular region into the center of the follicle, displacing resident B cells to the periphery of the follicle and forming the initial germinal center. At this stage the early germinal centers can be histologically identified (De Silva and Klein 2015). The displaced IgM^+ IgD^+ B cells form the 'mantle' that appears around developing secondary follicles (i.e., follicles with germinal centers).
- Day 5/6—Rapid proliferation of B cell blasts occurs.
- Day 7—Presence of light and dark zones in germinal centers signifies establishment of the mature germinal center. Dark zones are densely packed B cell blasts within an interconnected network of reticular cells that express CXCL12 (Bannard et al. 2013), while light zones consist of T_{FH} cells and follicular dendritic cells (Victora and Nussenzweig 2012).
- Several rounds of recirculation between dark and light zones, with proliferation and somatic hypermutation in dark zones and positive selection for high-affinity antibody production in light zones, results in a population of high-affinity memory B cells and plasma cells that eventually reside in the bone marrow.

Based on their histological characteristics, the cells of dark and light zones in germinal centers of humans were historically known as centroblasts and centrocytes, respectively. However, these morphological definitions apparently do not apply to

Fig. 7.4 (continued) 20× objective magnification. (**e**) This secondary lymphoid follicle in the spleen of a beagle dog has a central germinal center (*gc*) surrounded by a zone of follicular B cells (*f*). The marginal zone (*mz*) is visible but not as distinct as that seen in the rat. Note the extensive accumulation of eosinophilic erythrocytes in the interfollicular 'red pulp' (*). Blood engorgement of the spleen is a secondary effect of the barbiturate euthanasia agents that are used in dogs. The barbiturate causes relaxation of the smooth muscle trabeculae that normally maintain the somewhat constricted configuration of the spleen, thus allowing the spleen to fill with blood. The blood engorgement resulting from the barbiturate effect can hinder evaluation of the lymphoid component of the spleen of dogs. H&E stain, 10× objective magnification. (**f**) This secondary follicle in the mandibular lymph node of a beagle dog has a central germinal center (*gc*) surrounded by a follicular aggregation of B cells (*f*). Note the vascular supply to the follicle (*arrow*) that was fortuitously included in the histologic section. Expansion of the vascular supply to the lymph node is a critical element in the immunological response of lymph nodes. H&E stain, 13.9× objective magnification. (**g**) Brown-stained follicles are clearly demarcated in this immunohistochemical stain for B cells performed on a mesenteric lymph node of a Sprague-Dawley rat. CD45RA immunohistochemical stain with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 5× objective magnification. (**h**) Brown-stained T cell areas in the paracortex (*) are clearly demarcated in this immunohistochemical stain performed on a mesenteric lymph node of a Sprague-Dawley rat. CD3 immunohistochemical stain with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 3.82× objective magnification. (**i**) Brown-stained aggregates of proliferative cells are clearly demarcated in this immunohistochemical stain performed on a mesenteric lymph node of a Sprague-Dawley rat. Note the positively stained cells in medullary cords (*arrow*), indicating recent transmigration of proliferative cells from the germinal centers to the medullary sinuses. Ki67 immunohistochemical stain with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 2.02× objective magnification

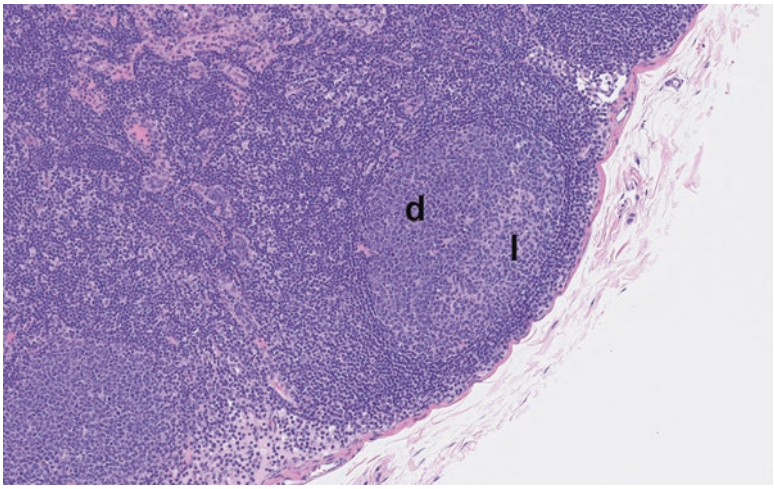

Fig. 7.5 This secondary follicle in the mandibular lymph node of a cynomolgus macaque has vague delineation of the dark (*d*) and light (*l*) zones that characterize lymphoid follicles in humans. The light and dark zones are variably visible in lymphoid follicles of nonhuman primates, but typically are not visible in lymphoid follicles of the remaining animal species used in non-clinical toxicology studies. H&E stain, 15× objective magnification

rodents. Studies of germinal center cell populations in mice did not reveal a difference in size or morphology of B cells in light versus dark zones, suggesting that B cells in germinal centers of rodents should be defined by their location, functional characteristics or immunophenotype (Victora and Nussenzweig 2012; Allen et al. 2007) rather than morphological features in routine histologic sections. By contrast, the germinal centers of nonhuman primates sometimes have discernible light and dark zones (Fig. 7.5).

Germinal centers are considered to be transient structures that have a finite lifespan, but there is little information regarding the duration of existence of individual germinal centers or processes involved in the involution of germinal centers.

See De Silva and Klein for a complete review of B cell dynamics in germinal centers (De Silva and Klein 2015), and Kurosaki et al. for a review of memory B cells (Kurosaki et al. 2015).

7.4.2 Secondary Evidence of Immune System Dysfunction

Microscopic changes in the primary and secondary immune system organs are the primary indicators of immune system effects, but it is also important to recognize lesions or microscopic changes that are indirectly related to xenobiotic effects on the immune system. These changes are commonly manifested as failures in the primary purpose of the immune system, i.e., protection against infectious agents. Although most moderate laboratory animal facilities have very strict standards of

hygiene and animal husbandry, non-clinical toxicology facilities typically are not germ-free. Chemically mediated immunosuppression may result in increased susceptibility to pathogens, resulting in outbreaks of specific diseases or a generalized increase in incidence of various infectious diseases. Outbreaks of specific diseases can present as a myriad of viral, bacterial, protozoan, mycotic, or parasitic diseases, some of which are transmitted between laboratory animals and animal handlers. Outbreaks of specific diseases are easily recognized, but recognition of a generalized increase in incidental infectious diseases or inflammatory lesions can be more problematic. In the latter situations, histologic alterations may involve a number of different organs, with no single organ being involved to a degree that results in an unequivocal treatment-related incidence pattern. As an example from one author's (GAP) experience, a standard non-clinical toxicology study in dogs had a low incidence of nonspecific inflammatory lesions in kidneys and other internal organs as well as probable demodex-related folliculitis in the skin of a few dogs in the xenobiotic-treated groups. The combination of inflammatory lesions in the various organs raised regulatory suspicion of an immunomodulatory effect of the xenobiotic, which resulted in a clinical hold on further development until a lengthy scientific review process was completed.

Microscopic changes associated with immunosuppresion are most commonly localized in organ systems that have direct contact with the external environment, principally the integumentary, respiratory, gastrointestinal and urinary systems. The respiratory and gastrointestinal systems are particularly susceptible to infections in immunocompromised animals. However, subtle increases in the incidence and/or severity of 'background' lesions such as nonspecific prostatitis in rats or exacerbations of *Demodex canis* infestation in dogs may be equally important as indicators of immune system modulation. These effects may involve changes in severity rather than prevalence, thus it is critical that all observations should be graded with regard to severity or quantified in some appropriate manner.

Detection of an increase in the incidence and/or severity of spontaneous disease processes must involve clinical observations and clinical pathology tests as well as histopathology, and should involve a 'whole animal' approach. Simply tabulating histopathologic lesions or clinical signs, or performing statistical analysis on clinical laboratory data, in the absence of this 'whole animal' approach may result in misinterpretation of xenobiotic-mediated immune system alterations.

7.4.3 Stress Response Versus Immunomodulation

Distinguishing stress-related responses from test article-related immunomodulation is a common dilemma in the interpretation of pathology data in toxicology studies. Interpretation of stress-related effects in non-clinical toxicology studies was the subject of a thorough review (Everds et al. 2013). Unfortunately, there is no test or parameter that is definitive evidence a stress response, therefore a conclusion that stress responses resulted in morphologic changes in lymphoid organs must be based on a combination of indicators and a weight-of-evidence approach.

The effects of stress on rats has been known since Hans Selye's first letter to *Nature* in 1936 (Selye 1936), and Selye's 'triad of stress' in rats was soon known to include enlargement of the adrenal glands, atrophy of the thymus and lymph nodes, and gastroduodenal erosions or ulcers (Szabo 1998, 2014; Szabo et al. 2012; Selye 1950). The changes in the thymus, and often the spleen, are frequently associated with lower organ weights at necropsy. Many of the stress-related histologic and hematologic changes are attributed to glucocorticoid release (Selye 1970). The glucocorticoid release associated with stress is known to affect all three major hematopoietic cell lines (lymphoid, myeloid and erythroid), and also promotes the differentiation of pre-adipocytes to adipocytes (Greenberger 1979; Laakko and Fraker 2002). The bone marrow effects are characterized histologically as a generalized reduction in cellularity accompanied by an increase in the adipocyte population. Stress-associated hematologic changes such as lower lymphocyte and eosinophil counts coupled with slightly higher neutrophil counts are known as the 'stress leukogram'. Glucocorticoid effects on circulating lymphocyte and eosinophil counts is such a consistent change that in earlier days it was the basis of the 'Thorn test' for adrenocortical function (Tucci et al. 1967; Molinatti et al. 1958). Despite this long-standing recognition of stress-related effects, there remains considerable difficulty in reliably distinguishing between direct immunomodulation and changes that are secondary to a stress reaction. The problem becomes even more acute when attempting to determine whether stress-related or test article-related alterations represent an adverse finding.

Glucocorticoid-mediated histologic changes in lymphoid organs can occur very quickly. Experimental administration of dexamethasone to mice, which one author (GAP) performed to produce positive control material for caspase IHC staining, resulted in a significant degree of cortical thymocyte apoptosis at 6 h following dexamethasone administration (Fig. 7.6a, b). At 12 h post-treatment there was severe diffuse apoptosis of cortical thymocytes (Fig. 7.6c, d), and by 24 h post-treatment there was almost complete loss of cortical thymocytes (Fig. 7.6e, f). The capacity of thymic macrophages in removing apoptotic thymocytes was equally impressive. At 24 h post-treatment the thymic cortex was nearly devoid of lymphocytes, and only a few clusters of tingible body macrophages laden with apoptotic debris remained to indicate the recent deluge of apoptotic activity. These observations based on experimental administration of glucocorticoids do not necessarily mirror the responses to physiologic levels of glucocorticoids, but reveal the glucocorticoid sensitivity and apoptotic machinery that is in place.

Stress responses are highly variable due to a number of reasons, including the type and duration of stimulus and the individual animal's ability to respond physiologically. Stress can be acute or chronic in nature. Non-clinical toxicology studies are designed to identify toxicities and by nature are stressful, particularly at high doses. An apparent dose-related occurrence of lymphoid depletion in the thymus, spleen and lymph nodes, with associated lower thymus and spleen weights but without the diagnostic support of an associated 'stress leukogram', can pose problems in interpretation in non-clinical toxicology studies. The problem is compounded when there is no secondary evidence of stress, i.e., there is no evidence of

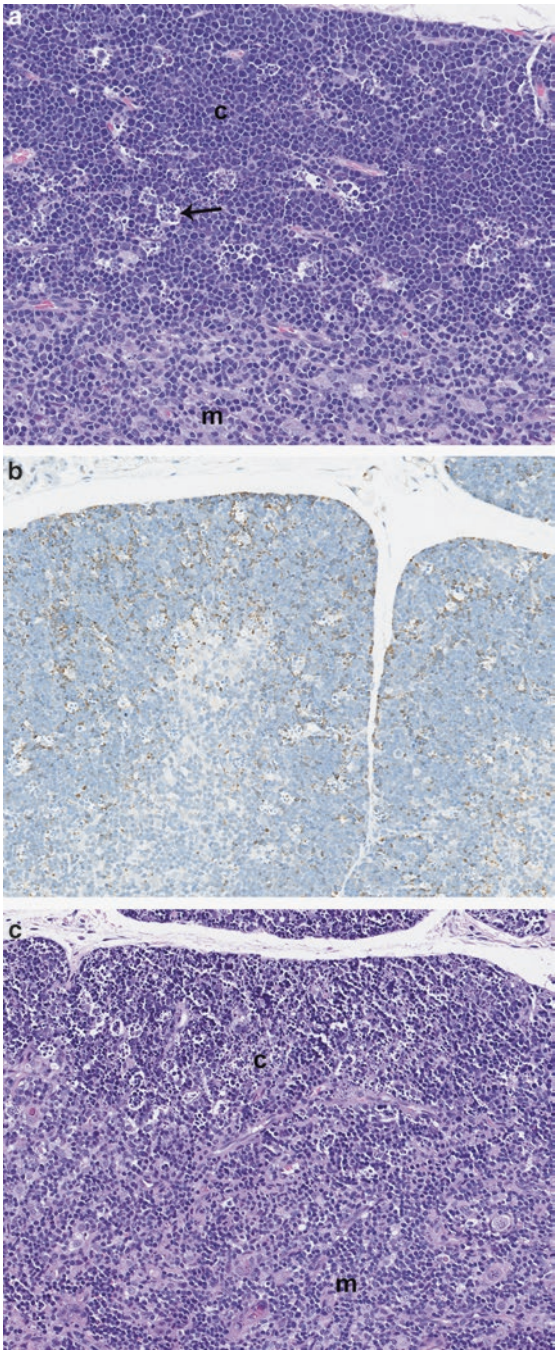

Fig. 7.6 (**a**) Apoptosis of lymphocytes in the thymic cortex (*c*) of a mouse occurs rapidly following experimental administration of corticosteroids. Note the clusters (*arrow*) of apoptotic cells and cell fragments 6 h following administration of dexamethasone. Developing thymocytes are most sensitive to glucocorticoid-induced apoptosis at the double-positive (DP) stage. Note the absence of apoptotic bodies in the medulla (*m*), indicating the absence of the corticosteroid-sensitive cell populations in the medulla. H&E stain, 40× objective magnification. (**b**) Immunohistochemical stain for

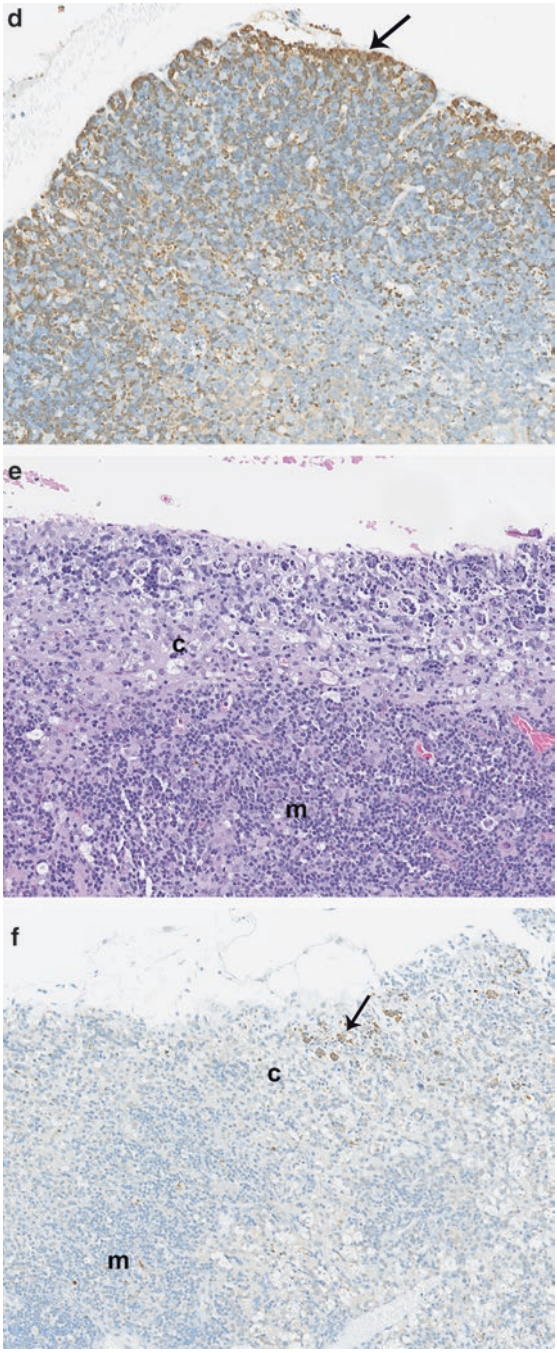

Fig. 7.6 (continued) activated caspase reveals positive (brown) staining of apoptotic cells and cell fragments in the thymic cortex at 6 h post-dexamethasone administration. Immunohistochemical staining for cleaved caspase 3 with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain. 20× objective magnification. (**c**) At 12 h following dexamethasone administration most cortical (*c*) thymocytes are densely basophilic and fragmented, indicating extensive apoptosis. Note sparing of the medulla (*m*), which indicates absence of the corticosteroid-sensitive double-positive

clinical alteration or change in habitus of the animals, no alteration in feed consumption, and no alteration in body weight gains. For more detailed information, see Everds et al. (2013).

Unfortunately, there is no readily available 'gold standard' assay that will support a definitive indication that a stress reaction existed in the animals. Serum corticosteroid levels have a very short half-life and are subject to multiple innate variables such as diurnal variation, therefore will not provide a statistically reliable indication of a stress response when based on the relatively small group size that is employed in typical non-clinical toxicology studies. There has been some interest in the use of urinary corticosterone levels for this purpose, in effect using the corticosterone accumulation in urine in the urinary bladder to average the circulating corticosteroid level (Thorpe et al. 2014; Jorgensen et al. 2013). While possibly of some utility when applied on a prospective basis, technical issues related to sample quantity, quality and storage parameters limit the usefulness of this assay when applied retrospectively.

Though of great interest in the field of toxicology, there has probably been even greater interest in stress effects in the field of behavioral science. This has resulted in a substantial body of literature on the topic, some of which may be pertinent to the problems encountered in toxicology. Various models have evolved for producing a stress response in animal species, some of which cannot be considered in view of current concerns regarding humane treatment of laboratory animals. However, some models such as the rodent restraint model remain acceptable. In this model, the rodent is placed into a cloth bag, e.g., the type used for restraining rats prior to decapitation, for approximately 2 h (Fachin et al. 2008). At the end of the restraint period the rodent is decapitated, blood collected for various assays, and the head with brain is preserved for histologic examination or various other laboratory analyses. Studies of this type have shown increased expression of proopiomelanocortin, a precursor of adrenocorticotrophic hormone (ACTH), in the anterior pituitary or corticotrophin releasing hormone (CRH) in the hypothalamic paraventricular nucleus are indicators of a stress response in rats (Noguchi et al. 2006; Yanagita et al. 2007). Demonstration of up-regulated expression of these molecules via immunohistochemistry or in-situ hybridization would could have promise as a confirmatory assay for stress response in rats, and perhaps other species. However, a

Fig. 7.6 (continued) (DP) thymocyte population. H&E stain, 20× objective magnification. (**d**) Immunohistochemical stain for activated caspase indicates apoptosis throughout the thymic cortex at 12 hours following dexamethasone administration, with a particularly intense band of apoptotic cells immediately beneath the surface capsule (*arrow*). Immunohistochemical stain for cleaved caspase 3 with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification. (**e**) At 24 h following dexamethasone administration the thymic cortex (*c*) has few remaining viable thymocytes, but a normal population of maturing thymocytes remains in the medulla (*m*). H&E stain, 20× objective magnification. (**f**) At 24 h post-dexamethasone the immunohistochemical for activated caspase reveals only clusters of degenerating thymocytes and apoptotic bodies (*arrow*) in the thymic cortex (*c*), with a normal population of non-apoptotic lymphocytes in the medulla (*m*). Immunohistochemical stain for cleaved caspase 3 with 3,3′-diaminobenzidine chromagen and hematoxylin counterstain, 20× objective magnification

number of questions must be answered by appropriate experimentation before such assays could be employed in regulated non-clinical toxicology studies. First and foremost is whether the increased molecule expression seen in response to the relatively short-term stress of behavioral studies is persistent through the longer duration of a standard toxicology study. One study showed that CRH expression in the paraventricular nucleus was altered following acute stress, but was unaltered after repeated stress for a period of seven days (Romeo et al. 2007).

7.4.4 Adverse Versus Non-adverse Determinations

Immunotoxicity has been defined as "any adverse effect on the components of and/or function of the immune system by a biological, chemical, or physical agent resulting from either direct or indirect actions and reflecting either permanent or reversible toxicity" (Hinton 2000), thus a determination of the adverse/non-adverse status of observations is central to defining the existence of immunotoxicity in toxicology studies. Despite the widespread application of this process over the past four decades, there is no universally accepted definitive of 'adverse' as it applies to non-clinical toxicology studies, and no standard guidelines exist for the designation of adverse effects in various organ systems. A number of definitions have been proposed for "adverse", with one of the most widely accepted being "change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other external influences"(IPCS 2004; Kerlin et al. 2015).

Questions have arisen regarding application of the phrases 'impairment of the capacity to compensate for additional stress', and 'increase in susceptibility to other external influences' to specific findings in toxicology studies. Those phrases would seem to justify extrapolation of study findings to situations that may involve additional stressors or external influences, i.e., studies conducted with differences in dosage levels, exposure duration, species of test animals, sex or strain of test animals, or various influences in the animal environment.

In an attempt to address some of these questions, the Scientific and Regulatory Policy Committee of the Society of Toxicologic Pathologists (STP-SRPC) organized a subcommittee to study the topic and propose standards and guidelines for use by individuals involved in the planning, conduct or interpretation of animal-based non-clinical toxicology studies. A recent publication (Kerlin et al. 2015) resulting from the subcommittee deliberations contained the following ten recommendations related to adversity considerations:

1. Adversity is a term indicating harm to the test animal.
2. The decision about whether or not test article-related effects (or a group of related effects) in a non-clinical study are considered adverse or nonadverse should be unambiguously stated and justified in subreports and/or the study report.

3. Adversity as identified in a non-clinical study report should be applied only to the test species and under conditions of the study
    a. When toxicity in a test animal is interpreted as being specific to that species and lacking relevance to humans, the test article effect may still be an adverse response for the species being tested.
    b. Neither the therapeutic indication nor the patient population should influence adversity decisions in the test species.
    c. Test article-related exacerbations of background lesions in animals can be considered adverse.
4. Toxic effects on cells, tissues, organs, or systems within the test animal should be assessed on their own merits.
    a. Effects believed to be suprapharmacological should be considered either adverse or nonadverse.
    b. All relevant effects should be considered in the interpretation of adversity, regardless of whether they are perceived to be primary, secondary, or tertiary.
    c. Responses interpreted as being "adaptive" should be considered either adverse or nonadverse.
5. Communication of what is considered adverse and assignment of the NOAEL in the overall study report should be consistent with, and supported by, the information provided in the study subreports.
    a. Test article-related changes should be documented in subreports, regardless of whether they are considered to be adverse or nonadverse.
    b. Test article-related adverse findings considered to be part of a constellation of related effects should be discussed together.
    c. The NOAEL is to be assigned for the study as a whole rather than to subreports.
    d. Value statements such as "not biologically relevant" or "not toxicologically important" should only be used in the study report when defined and supported by a sound rationale.
6. Communication of adverse findings and the NOAEL should include direct interaction between staff within different contributing scientific disciplines.
7. The NOAEL for a test article should be communicated in an overview document based upon data from multiple studies.
8. In order to place them in appropriate context, the use of NOAELs in data tables should be referenced to explanatory text.
9. Nonclinical scientists, including toxicologists, pathologists, and other contributing subject matter experts who interpret data from nonclinical studies, should be active participants in assessing and communicating human risk.
10. All available data from all nonclinical studies must be evaluated together to define any potential toxicities and to predict human risk.

The STP-SRPC recommendations make it clear that adversity decisions in a nonclinical toxicology study should be based solely on the observations in the individual study, with no speculation of what might result from different study designs or the impact of additional variables. In order to avoid confusion on the part of readers, it is good practice for the pathology report to state that determinations of adversity in an individual report are made solely within the context of the reported study, and apply to animals of an indicated species, strain, gender, and age when given a specified dose/dosage level via a specified route and duration of administration. Nothing further is implied.

The interpretation of histologic alterations in lymphoid organs as adverse/nonadverse can be difficult, as lymphoid organs are not histologically static throughout life. Progressive involution of the thymus begins at or near sexual maturity and progresses at different rates in individual species. The bone marrow has fewer age-related changes than the thymus, but tends to become less cellular and contains more adipocytes with aging. Some secondary lymphoid organs (lymph nodes, spleen and Peyer's patches) undergo age-related changes (see Volume 1, Chap. 5) and, in addition, have major histologic alterations based on the antigen load that is presented to the organ. Interpretation of histologic changes in secondary lymphoid organs can become even more difficult when test article- or stress-related immunomodulation results in infectious or inflammatory disease processes that, in turn, cause reactive changes in the secondary lymphoid organs. Depending on the type and level of the original xenobiotic- or stress-related immunomodulation, and the contribution of additional complicating factors, the changes in secondary lymphoid organs may be simple or complex. These difficulties in interpretation are best approached from a 'whole animal' or 'whole study' perspective, as opposed to detailed study of histologic changes in individual immune system organs (Kimber and Dearman 2002).

7.5 Special Circumstances in the Pathology Evaluation

7.5.1 Studies Conducted in Juvenile Animals

Interpretation of histologic changes in juvenile toxicology studies is particularly complex. In addition to the direct, indirect, nutrition- and stress-related histologic alterations that are encountered in non-juvenile toxicology studies, juvenile toxicology studies may also have xenobiotic-associated alterations in postnatal organ development. Postnatal development of the immune system, in particular, is partially driven by environmental factors, as opposed to the genetically determined histologic development of most other organ systems.

Challenges in histopathologic interpretation in juvenile toxicology studies are most apparent when dealing with observations in unscheduled death animals, where age- and sex-matched concurrent control animals typically are not available for comparison. In these cases, histologic abnormalities in tissues of decedent rats may be (a) normal for the age and state of development, (b) an indication of a direct effect of the xenobiotic, (c) hindered organ development due to xenobiotic influences, (d) a manifestation of stress-related effects, or (e) some combination of these

influences. Published information regarding the postnatal development schedule of various tissues of laboratory animals is evolving (Picut et al. 2014, 2015a, b; Parker et al. 2015; Parker and Picut 2016), but remains generally sparse. When evaluating histologic observations in decedent juvenile animals, pathologists and toxicologists are often dependent on knowledge and experience to distinguish between direct or indirect xenobiotic-related effects versus normal postnatal development. If extensive involvement in studies involving juvenile animals is anticipated, consideration should be given to establishing a library of histologic specimens at various postnatal periods for comparison with tissues from decedent animals. This is an ideal use for scanned digital slides, which are easily shared between multiple users. Optionally, and perhaps ideally, study protocols for toxicology studies that involve juvenile animals would include additional naïve control animals that could be euthanized concurrently with decedents, thus providing a relevant biological control to aid in differentiating normal postnatal organ development from xenobiotic influence.

7.5.2 Studies Involving Biologics

As used herein, 'biologics' refers to molecules that are prepared through the use of biological methods, commonly mammalian or plant cells, bacteria, insects, or yeast (Leach 2013). The resultant products are commonly proteins, but may be nucleic acids, carbohydrates or a combination of multiple types of molecules (FDA 2015). Biologics are included within the FDA definition of 'drugs' and are generally covered by the same laws and regulations, but differences exist regarding their manufacturing processes (FDA 2015). Biologics commonly fall into general functional categories of monoclonal antibodies, cytokines, growth factors, enzymes, and peptides that are focused on specific molecular targets (Leach et al. 2014). In each category, the biologic may target the signaling or effector molecule or its receptor. Biologics commonly are large molecules, e.g., approximately 150 kD molecular weight for monoclonal antibodies (Delves et al. 2011), while traditional 'small molecule' therapeutics typically are less than 500 daltons in molecular weight (Leach 2013). Pathology interpretations of biologic-associated changes can be particularly complex because (a) the desired therapeutic effect of the biologic may include immunomodulation and (b) the biologic may stimulate an immune response over the time course of a preclinical study. The immune response may have multiple effects that include reducing (rarely increasing or prolonging) the physiological activity of the biologic, altering the function of endogenous molecules, or producing hypersensitivity reactions to the biologic (Leach 2013).

Biologics tend to be relatively innocuous molecules that are not expected to have direct toxic effects on organ systems. However, that does not preclude the possibility of clinical pathology alterations or histopathological observations associated with administration of biologics. For example, intravenous administration of any type of particulate material may result in clumping and eventual destruction of platelets (Cohen et al. 1965), with the expected effect on platelet counts. Administration of oligonucleotides is known to be associated with accumulations of basophilic particles in proximal tubular epithelial cells of the kidney, which are more readily visualized

with toluidine blue staining than routine hematoxylin and eosin staining (Monteith et al. 1999). Intravenous administration of peptides may result in accumulations of microscopically visible peptide aggregates within macrophages near the injection/infusion site as well as within macrophages at distant sites such as lymph nodes, Kupffer cells of the liver and mesangial cells of renal glomeruli.

Biologics are typically generated in a species other than the intended patient population, and the non-clinical test system typically involves species other than the intended recipients of the final product. The laboratory animals used in non-clinical studies would be expected to mount some form of immunological response to the presence of these 'non-self' molecules but, in general, these responses are not interpreted as evidence of 'toxicity' that is relevant to human risk. By contrast, evidence of biologic-associated adverse effects that are related to the intended pharmacologic action or off-target effects of the biologic may have direct implications for human risk.

Reactions to biologic commonly involve some form of immunological hypersensitivity. The original Gell and Coombs classification of hypersensitivity postulated four general mechanisms of immune-mediated tissue injury:

- *Type I* (anaphylactic) reactions are immediate hypersensitivity reactions mediated by IgE interaction with antigen, with subsequent release of histamine and other mediators from mast cells and eosinophils.
- *Type II* (cytotoxic) reactions are antibody-mediated reactions caused by antibody-antigen interactions on cell surfaces.
- *Type III* (immune complex) reactions result from formation of circulating immune complexes and their deposition in tissues.
- *Type IV* (cell-mediated) reactions are delayed hypersensitivity reactions initiated by sensitized T lymphocytes.

Type IV reactions have subsequently been subdivided into four subcategories (Pichler 2007):

	Type IVa	*Type IVb*	*Type IVc*	*Type IVd*
Immune reactant	IFNγ, TNF-α (Th1 cells)	IL-4, IL-5/IL-13 (Th2 cells)	Perforin, granzyme B, FasL (CTL)	CXCL8, GM-CSF (T cells)
Ag presentation	APCs	APCs	Target cells	APCs
Effector cells	Macrophages	Eosinophils	$CD8^+$ T cells	Neutrophils
Examples	Tuberculin reaction	Asthma, allergic rhinitis	Contact dermatitis Stevens Johnson syndrome Toxic epidermal necrolysis	Acute generalized exanthematous pustulosis Behcet's disease

Modified from Pichler (2007)

Type I and Type III reactions are most commonly encountered in nonclinical toxicology studies, and each reaction typically involves formation of anti-drug antibodies (ADA) (Leach et al. 2014). Type I reactions commonly involve mast cell degranulation and infiltrations of eosinophils in affected tissues. Mast cell degranulation may be difficult to document unless mast cells with reduced number of cytoplasmic granules can be demonstrated via immunohistochemical staining or transmission electron microscopy. Eosinophil infiltrates are usually apparent in routinely stained histologic sections, though in some species and some tissues it may be difficult to distinguish eosinophils from degenerating neutrophils. In those cases, a tissue Giemsa stain may be helpful.

In addition to the originally proposed mechanism for induction of type I reactions, mice and humans have an additional induction mechanism that involves binding of ADAs to FC receptors on surface of neutrophils (Jonsson et al. 2012). A second exposure to antigen results in cross-linking of the FC receptors, with activation of neutrophils and release of platelet-activating factor (PAF), which is much more potent than histamine in initiating an anaphylactic response (Jonsson et al. 2011).

Type III reactions are based on interactions between circulating antigens and antibodies (biologics and ADAs, respectively). When ADAs are formed in response to circulating antigens, some level of interaction between the antigens and antibodies is likely. When ADAs and antigen molecules are present in the correct stoichiometric ratios, i.e., when antigen is only slightly in excess of antibody levels, the resulting circulating antigen-antibody complexes may be too small for recognition and removal by the fixed macrophage system, yet large enough to lodge in certain tissues and activate complement (Leach et al. 2014; Snyder 2007). Tissues with a particular predilection for immune complex deposition include ‘filtration sites’ such as the synovial apparatus of joints, ciliary bodies of the eyes, choroid plexus of cerebral ventricles, and renal glomeruli (Snyder 2007). Vascular structures that have interrupted laminar blood flow (e.g. postcapillary venules) or turbulent blood flow (e.g. arterial branch-points or cardiac valves) may also be predisposed to immune complex deposition (Leach et al. 2014). It should be noted that hepatic sinusoids have many features in common with postcapillary venules, including loss of laminar blood flow, plus sinusoidal endothelial cells have Fc receptors that bind immune complexes (Smedsrod et al. 1985; Lovdal et al. 2000). Presence of immune complexes in tissues results in activation of complement, with elaboration of factors such as C5a that attract neutrophils and macrophages to the site of immune complex deposition (Snyder 2007). Activated neutrophils and macrophages produce pro-inflammatory mediators, including vasoactive amines that promote penetration of immune complexes into the wall of blood vessels. In some situations the end result is the hallmark histopathological change, which consists of fibrinoid necrosis of the tunica media of arteries and arterioles.

For additional information, see reviews and book chapters by Leach and Snyder (Snyder 2007, 2012; Leach 2013; Leach et al. 2014).

7.5.3 *Immunomodulatory Experimental Procedures and Vehicles*

7.5.3.1 Infusion Studies

Nonclinical toxicology studies may involve intravenous dose administration and, particularly for repeated dose studies, commonly involves vascular catheterization. Some level of nonspecific inflammation is commonly associated with placement of vascular catheters (Fig. 7.7b) (Gleason and Parker 2013), and the secondary effects of inflammation must be considered in the interpretation of potential immunomodulatory effects of the test article. Localized inflammation associated with catheter placement commonly influences hematology test parameters, resulting in an 'inflammatory hemogram', and may influence some aspects of the clinical chemistry and coagulation analysis.

Of particular concern to those involved in interpretation of non-clinical toxicology studies is the plethora of systemic alterations included in the 'acute phase reaction' to inflammation. Acute inflammation in mammals is associated with a transient increase in a group of circulating proteins that are collectively known as acute phase proteins, which are largely proteins produced by the liver in response to cytokine stimulation. The group includes serum amyloid A protein (SAA), fibrinogen, C-reactive protein (CRP), haptoglobin, complement factors C3 and C9, hemopexin, ceruloplasmin, $_{\alpha 2}$-macroglobulin, CD14, $_{\alpha 1}$-antichymotrypsin (ACT), $_{\alpha 1}$-cysteine proteinase inhibitor ($_{\alpha 1}$CPI), and $_{\alpha 1}$-antitrypsin (AAT) (Bain 1994). Circulating acute phase proteins are responsible for many of the systemic effects of inflammation, which are largely aimed at preparing the body for resistance to systemic invasion and facilitating local resistance to pathogens. The acute phase proteinase inhibitors (e.g. AAT, ACT, $_{\alpha 1}$CPI, and $_{\alpha 2}$M) reduce tissue damage due to proteinases that may be released by dead or dying cells. Hemopexin and haptoglobin bind to heme and globin, respectively, which may be released by erythrolysis in inflammatory lesions. Serum amyloid A protein and CRP have functions which suggest a scavenger role, but no single function has been identified that would explain the marked increase of these serum proteins in an acute phase reaction (Agrawal et al. 1993).

Some serum components are known as negative acute phase reactants, i.e., production of these components typically decreases in response to inflammation. For example, serum albumin levels are commonly decreased as part of the systemic reaction to inflammation. This negative acute phase response affects not only the directly measured serum albumin levels, but also the calculated albumin: globulin ratio that is considered in the overall evaluation of immunomodulation. Lower serum albumin in the rat results in lower serum calcium-binding capacity, with the end result that serum calcium levels may be lower as a nonspecific response to inflammation.

C-reactive protein (CRP) is known to opsonize particles such as microbes, activate complement, bind to IgG receptors on mammalian cells and phosphocholine in bacterial membranes, and recognize nuclear constituents in damaged cells. Serum

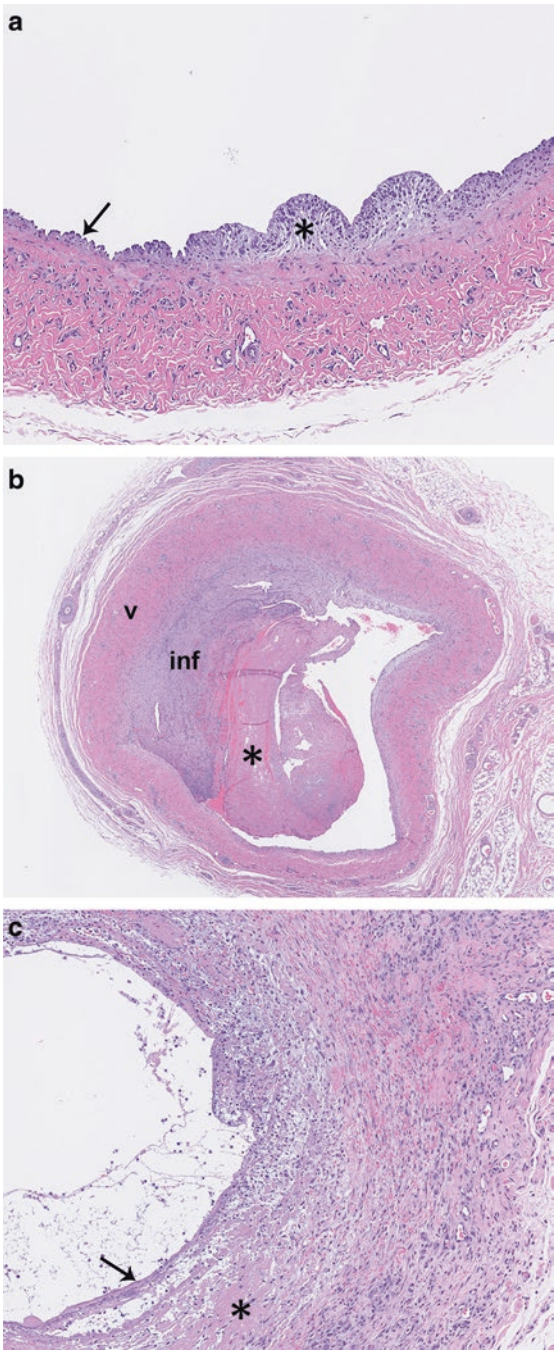

Fig. 7.7 (**a**) The catheterized vein from a non-dosed Sprague-Dawley rat shows mild intimal and subintimal reaction (*) associated with presence of the catheter. Note the normal-appearing endothelium and intima (*arrow*) adjacent to the thickened reactive area. H&E stain, 10× objective magnification. B The catheterized vein from a non-dosed Sprague-Dawley rat has a large segment in which the endothelium and intima are replaced by a thick layer of infiltrating inflammatory cells and tissue debris (*inf*). Note the thick layer of fibrinous debris (*) overlying the area of inflammation. The pathologic process does not penetrate through the wall of the vein (*v*) into the surrounding tissue. H&E stain, 2.5× objective magnification. (**c**) The catheterized vein from a non-dosed Sprague-Dawley rat has loss of endothelial cells (*arrow*) and subintimal accumulation of necrotic debris (*). Inflammatory cell infiltration extends through the wall of the vein into the perivascular tissue at the right margin of the image. H&E stain, 10× objective magnification

CRP level has been shown to be a reliable indicator of inflammation associated with atherogenesis in humans (Dupuy et al. 2003), but serum CRP level is not a reliable indicator of inflammation in all species. Serum haptoglobin level has been shown to be a better indicator of systemic inflammation in swine, and serum levels of $_{\alpha 2}$–macroglobulin, haptoglobin or fibrinogen are more accurate than CRP levels as indicators of inflammation in laboratory rats (Dasu et al. 2004; Chen et al. 2003; Giffen et al. 2003).

The acute phase response is generated when focal injury at an extrahepatic location prompts local macrophages to release a first wave of cytokines that includes IL-1, tumor necrosis factor alpha (TNFα), and a small amount of IL-6. Absorption of the first wave of cytokines into surrounding cells is followed by a second wave cytokine release, including a large amount of IL-6 that promotes massive production of acute phase proteins by hepatocytes (Bain 1994). IL-6 is the prototype signaling molecule in the induction of the acute phase response, but IL-22, which is produced by activated DCs and Th cells, also has the ability to up-regulate production of acute phase proteins (Nagalakshmi et al. 2004). Presence of these signaling and effector molecules that result from the inflammation associated with catheter placement or other experimental manipulation can be a significant complication in the interpretation of xenobiotic-related immunomodulation.

Production of acute phase proteins has historically been considered a function of hepatocytes, but recent evidence indicates a similar spectrum of proteins is produced in the uterus and mammary gland during pregnancy and lactation, and may be particularly accentuated during the involution period that follows following parturition and lactation (Hayashida et al. 1986; Stein et al. 2004). These latter observations have obvious implications for developmental and reproductive toxicology (DART) studies.

7.5.3.2 Saline Infusion or Injection

Saline or saline-based vehicles are commonly used for intravenous administration of xenobiotics. Administration of saline to rats at the rate of 40 or 80 mL/kg for 30 consecutive days was associated with periarterial infiltrations of eosinophils in the lungs of rats (Morton et al. 1997). Eosinophil infiltration has been classically considered a hallmark histologic feature of allergic reaction or metazoan parasitism, but the possibility of saline-associated eosinophil infiltration must be considered when periarteriolar eosinophil infiltrations are observed in the lungs of rats.

7.5.3.3 Polyethylene Glycol-Conjugated Test Articles

Covalent binding to one or more polyethylene glycol (PEG) molecules to biopharmaceuticals can extend plasma half-life, improve stability, and sometimes decrease the immunogenicity of therapeutic agents (Ivens et al. 2015). Cellular vacuolation associated with the PEG component may be seen in a variety of cells, particularly macrophages and macrophage-derived cells (Rudmann et al. 2013). The

vacuolation of phagocytes is considered to be a reflection of the normal, non-adverse physiological function of phagocytes if there is no distortion of normal tissue structures, no evidence of degeneration, inflammation or necrosis, no impairment of function and no changes in biomarkers of injury (Ivens et al. 2015). Vacuolation of parenchymal or interstitial cells is considered to be a non-adverse histologic finding if there are no associated adverse changes such as necrosis, degeneration, or inflammation, no changes in biomarkers of injury, and no evidence of impaired function (Ivens et al. 2015). For more detailed information, see reviews by Ivens and Rudman (Ivens et al. 2015; Rudmann et al. 2013).

7.5.4 Immunomodulation Due to Concurrent Disease

Interaction between spontaneous disease and the immune system is bidirectional, as immunomodulation can promote the development of various pathologic processes and the development of spontaneous pathologic processes can result in immunomodulation. Thorough familiarity with the pathogenesis of spontaneous diseases as well as familiarity with immunology is helpful in the investigation of these occurrences. Infectious and parasitic diseases are uncommon in laboratory rodents that are obtained from reputable sources and maintained in accordance with currently accepted management practices, though a few infectious diseases and parasitic infestations remain despite the best attempts at eradication. Infectious agents and parasitic infestations are more commonly encountered in nonhuman primates (Sasseville and Diters 2008; Haley 2012; Olivier et al. 2010; Abbott and Majeed 1984), where they may pose significant challenges to interpretation and development decisions in studies that typically involve small numbers of animals per group. In some cases the immunological effects of disease agents are partially known, e.g., the cytokine perturbations associated with *Syphacia* infestation in rodents, but in most instances the immunological ramifications of the spontaneous diseases are unknown or speculative. Viral, bacterial, fungal and parasitic diseases that may complicate toxicology studies in nonhuman primates are summarized in Table 7.5 (modified from Haley 2012).

Chronic inflammatory lesions may be associated with alterations in leukogram, clinical chemistry parameters, cytokine expression, and activation status of various cellular components of the innate and adaptive immune systems. Presence of such lesions introduces a significant uncontrolled variable into toxicology studies, and may serve to obscure or enhance xenobiotic-related toxicologic injury. Brief presentations of some of the more common inflammatory lesions follows.

7.5.4.1 Auricular Chondritis

Inflammation centered on the central cartilage of the pinna (auricular chondritis/chondropathy) (Fig. 7.8) is a known result of immunization to type 2 collagen (Fujiyoshi et al. 1997; Cremer et al. 1981; McCune et al. 1982), and is recognized

Table 7.5 Selected pathogens of nonhuman primates used in toxicology studies

Viral agents	Bacterial agents	Fungal agents	Protozoan & metazoan parasites
Adenovirus	Campylobacter	Aspergillus spp.	Acanthamoeba spp.
Cercopithecine herpesvirus I (B virus)	E. coli, enteropathogenic	Candida albicans	Balantidium coli
Cynomolgus polyomavirus	Helicobacter pylori	Dermatophyte spp.	Blastocystis spp.
Cytomegalovirus	Helicobacteri heilmannii-type		Cryptosporidium spp.
Hepatitis A virus	Lawsonia		Cyclospora spp.
Lymphocryptovirus	Moraxella catarrhalis		Demodex spp.
Measles virus	Mycobacterium tuberculosis		Endolimax nana
Polyomavirus	Rhodococcus equi		Entamoeba coli
Rhesus rhabdovirus	Salmonella spp.		Enterocytozoon bieneusi
Simian immunodeficiency virus	Shigella spp.		Giardia duodenalis
Simian parvovirus	Yersinia spp.		Oesophagostomum spp.
Simian type D retrovirus			Plasmodium spp.
Simian varicella virus			Pneumonyssus semicola
Simian virus 40			Sarcocystis spp.
			Schistosoma spp.
			Strongyloides fulleborni
			Toxoplasma gondii
			Trichomonas spp.
			Trichuris trichiura

Modified from Haley (2012) Toxicol Pathol 40: 261–266

as a spontaneous disease entity in laboratory rats (Prieur et al. 1984; Miller et al. 2008). There is evidence that auricular chondritis in rodents is associated with placement of metal identification tags in the pinna (Kitagaki and Hirota 2007; Kitagaki et al. 2003; Miller et al. 2008). Auricular chondritis associated with unilateral ear tagging often is bilateral, indicating a systemic effect that presumably is of immunologic origin (Meingassner 1991; Miller et al. 2008). Ear tag identification systems would seem to be specifically contraindicated in experimental models involving immunity to collagen, e.g., collagen-induced arthritis models.

7.5.4.2 Inflammatory Foot Lesions in Rats

Ulcerative and inflammatory lesions on the footpads (‘tarsal granulomas’) of rats are a common occurrence, particularly in studies of prolonged duration. The lesions typically involve ulceration of the footpad, most commonly involving the rear limbs, with associated severe inflammatory cell infiltration (Fig. 7.9a). Regional lymph

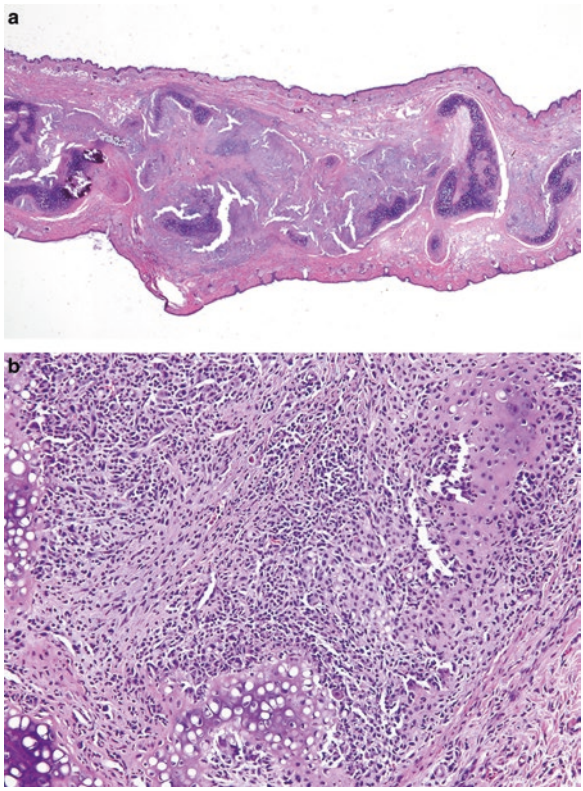

Fig. 7.8 (**a**) This section of the pinna (ear flap) of a Sprague-Dawley rat shows fragmentation of the central cartilage, with associated inflammatory cell infiltration. H&E stain, 2.5× objective magnification. (**b**) A severe mixed inflammatory cell infiltration is associated with cartilage fragments in auricular chondropathy in a Sprague-Dawley rat. H&E stain, 20× objective magnification

nodes commonly have pronounced reactive changes (Fig. 7.9b). Conventional wisdom has suggested the incidence of tarsal granulomas is higher in rats maintained in wire-bottom rather than solid-bottom cages, based on the suspicion that the greater body weight of aged rats leads to foot injury from the cage wire. However, in a recent investigation of the relationship between tarsal granulomas and colony management practices in rats from two-year carcinogenicity studies, there was evidence that the incidence of tarsal granulomas was more related to final body weight than to wire-bottom versus solid-bottom cages, and that final body weight was influenced by pair housing versus individual housing (Zhao et al. 2015). A study of rats housed on wire-bottom versus solid-bottom plastic cages indicated no difference in clinical pathology parameters between the two groups (Sauer et al. 2006).

7.5.5 Syphacea Infestation

Pinworm (*Syphacea* and *Aspicularis* spp.) infestation has long been common in rodent colonies, and remains a problem in modern rodent colonies (Clifford and Watson 2008). These nematode parasites have not been considered significant

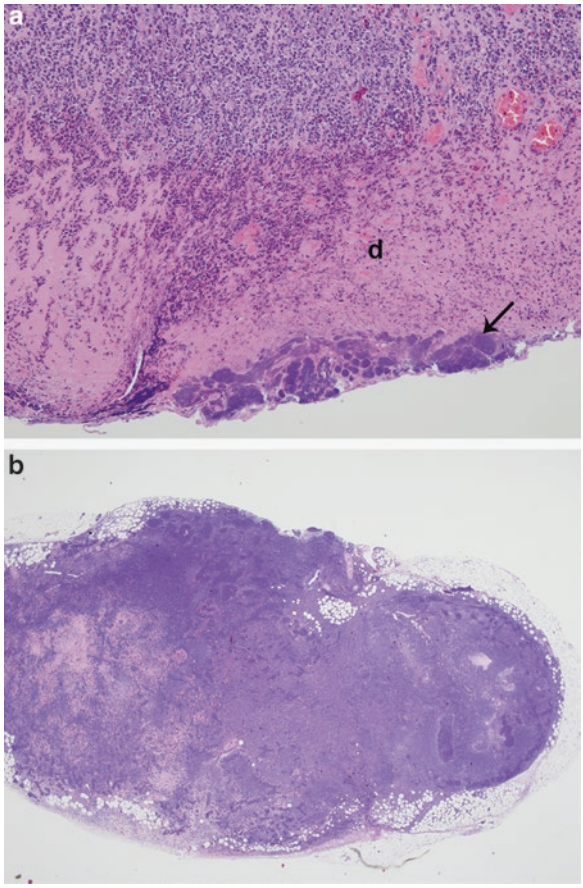

Fig. 7.9 (**a**) The ulcerated footpad from a non-dosed male Wistar rat has a thick surface layer of cellular debris (*d*) with prominent bacterial colonization (*arrow*). Lesions of this type typically do not resolve, and may become so extensive as to necessitate euthanasia of the rat. An inflammatory lesion of this magnitude and duration may have an effect on the general cytokine milieu, thus could influence test article-associated immunologic reactions. H&E stain, 20× objective magnification. (**b**) The iliac lymph node from the rat shown in Fig. 7.9 is hypercellular and has extensive reactive changes secondary to the footpad ulceration. H&E stain, 4× objective magnification

pathogens for the parasitized rodents, however, there is evidence that pinworm infestation is associated with alterations in immune responsiveness which could potentially modify responses to xenobiotics or biologics. Helminth parasites induce strong immune responses that are initiated by cytokines, particularly IL-4 and IL-13 (Maizels and Holland 1998). It has been shown that expulsion of the nematode parasite *Nippostrongylus braziliensis* from mice requires signaling via IL-4Rα and Stat6, and that IL-13 may be more important than IL-4 as an inducer of the Stat6 signaling that leads to worm expulsion (Urban et al. 1998). Experimental infection of BALB/c mice with *Syphacea obvelata* induced a transient T_H2-type response with elevated IL-4, IL-5 and IL-13 production as well as production of parasite-specific IgG1 (Michels et al. 2006). BALB/c mice that were deficient in IL-13, IL-4/13, or the IL-4Rα chain developed chronic disease after experimental infection with *Syphacea obveleta* and had greater than 100-fold higher parasite burden, increased gamma interferon production, parasite-specific IgG2b, and a Th2 response (Michels et al. 2006). Mice deficient in IL-4 alone had only slightly higher parasite burden than control mice, suggesting that

IL-13 plays the dominate role in control of *Syphacea obveleta* infestation in mice (Michels et al. 2006). *Syphacea obveleta*-infested mice immunized against ovalbumin had more severe anaphylactic shock and reduced IL-4 and IL-5 than non-infected control mice (Michels et al. 2006). Given the complex interactions of immune system signaling pathways, these parasite-associated cytokine perturbations would be of particular concern in studies of xenobiotics with therapeutic immunomodulatory effects.

Pinworm infestation to some extent opposes the immunological deficiency in athymic nude mice. Antigenic stimulation of athymic mice on the BALB/c background by infection with *Aspiculuris tetraptera* and *Syphacia obvelata* induced a proliferation of T- and B-lymphocytes in spleen and lymph nodes, with occasional germinal center formation that suggests an intact humoral response. The T-lymphocytes showed a greater expression of the Thy-1 marker than was seen in non-parasitized control mice, and the T cells were able to mount a response to sheep erythrocytes and other thymus-dependent antigens (Beattie et al. 1981). Athymic nude mice typically have no detectable immunological response to sheep erythrocytes, but athymic mice with pinworm infestations commonly have proliferations of lymphoid cells that have basic characteristics of T cells, and the cellular proliferations may progress to overt lymphoid neoplasia (Baird et al. 1982). One could question whether these parasite-induced abrogations of the athymic immune status could involve the extrathymic T cell generation seen in the gastrointestinal tract of aged normal mice after thymic involution occurs (Abo 1993, 2001).

In addition to effects on specific immunity, there is evidence that pinworm infestation affects hematopoiesis. *Syphacia* obvelata infestation in mice is known to be associated with increased myelopoiesis and erythropoiesis, as well as altered reactivity of bone marrow hematopoietic precursors to IL-17, sustained phosphorylation of multiple mitogen-activated protein kinases (MAPKs), and enhanced expression of inducible nitric oxide synthase (iNOS) in bone marrow cells (Ilic et al. 2010; Bugarski et al. 2006).

There are fewer reported studies of *Syphacia*-related complications in rats, but it seems reasonable to assume that rats have reactions that are similar to mice. Studies in male Wistar rats have shown that rats with pre-existing infestations with *Syphacia muris* or *Aspiculuris tetraptera* have significantly greater serum levels of TNFα, IL-10 and IgE than non-infested rats at 21 days following ovalbumin sensitization (Demirturk et al. 2007).

7.5.6 CAR Bacillus Infection

The cilia-associated respiratory bacillus (CAR bacillus) was first reported in aging rats with chronic respiratory disease, which was initially attributed to murine mycoplasmosis, but subsequent investigations have shown that CAR

bacillus alone can produce substantial pulmonary lesions in rats (Cundiff 1992; van Zwieten et al. 1980; MacKenzie et al. 1981; Ganaway et al. 1985). Similar CAR bacillus-associated pulmonary lesions have been reported in wild rats, mice, rabbits and the African white-tailed rat (*Mystromys albicaudatus)* (Cundiff 1992). CAR bacillus organisms colonize the respiratory epithelium, and may be present in one- to two-week-old pups born to CAR bacillus-positive dams (Ganaway et al. 1985). Histologic changes in the lung include pronounced BALT hyperplasia (Fig. 7.10b) and mild hyperplasia of ciliated epithelial cells in airways (Cundiff 1992). Numerous bacilli can be demonstrated within the ciliated surface by use of Warthin-Starry or Grocott methenamine silver stains (Fig. 7.10c), or immunohistochemical staining (Ganaway et al. 1985; Itoh et al. 1987). Presence of CAR-bacillus-associated BALT hypertrophy/hyperplasia may complicate interpretation of immunomodulatory effects of xenobiotics, particularly when lung changes conflict with observations in other immune system organs. The organisms are not readily visualized in routinely stained histologic sections, therefore, special stains for organisms are indicated in any situation that involves unexplained BALT prominence in rats. Murine mycoplasmosis infection of the lung must be considered as a differential diagnosis, though the incidence of murine respiratory mycoplasmosis has declined in recent decades (see discussion below).

7.5.7 Murine Mycoplasmosis

Murine respiratory mycoplasmosis (chronic respiratory disease-CRD) was a major infectious disease that substantially reduced long-term survival of laboratory rats prior to the introduction of management practices that reduced the incidence of CRD. The fully developed pulmonary lesion typically includes chronic active inflammation, bronchiectasis, and marked expansion of BALT (Fig.7.11b). Elucidation of the pathogenesis of CRD resulted in colony management alterations that would help limit the clinical expression of the disease (Broderson et al. 1976; Lindsey and Cassell 1973; Lindsey et al. 1971; Cassell et al. 1973), and thereafter commercial suppliers began offering stock that were free of the causative organisms (Weisbroth et al. 2006). However, murine respiratory mycoplasmosis remains an occasional complication of experimental results, as evidenced by recent reports showing that mycoplasma-associated BALT proliferation in the lungs of rats was misinterpreted as pulmonary lymphosarcoma in carcinogenicity studies (Caldwell et al. 2008; Schoeb et al. 2009; Schoeb and McConnell 2011a, b; Goodman et al. 2009). While the BALT expansion seen with murine respiratory mycoplasmosis can be quite florid, resembling a neoplastic proliferation, differentiating features of mycoplasmosis include concurrent inflammatory lesions in the lung and middle ear, and restriction of the mycoplasma-associated lymphoid proliferation to the lungs rather than the disseminated lymphoid tissue involvement that is typically seen with lymphosarcoma.

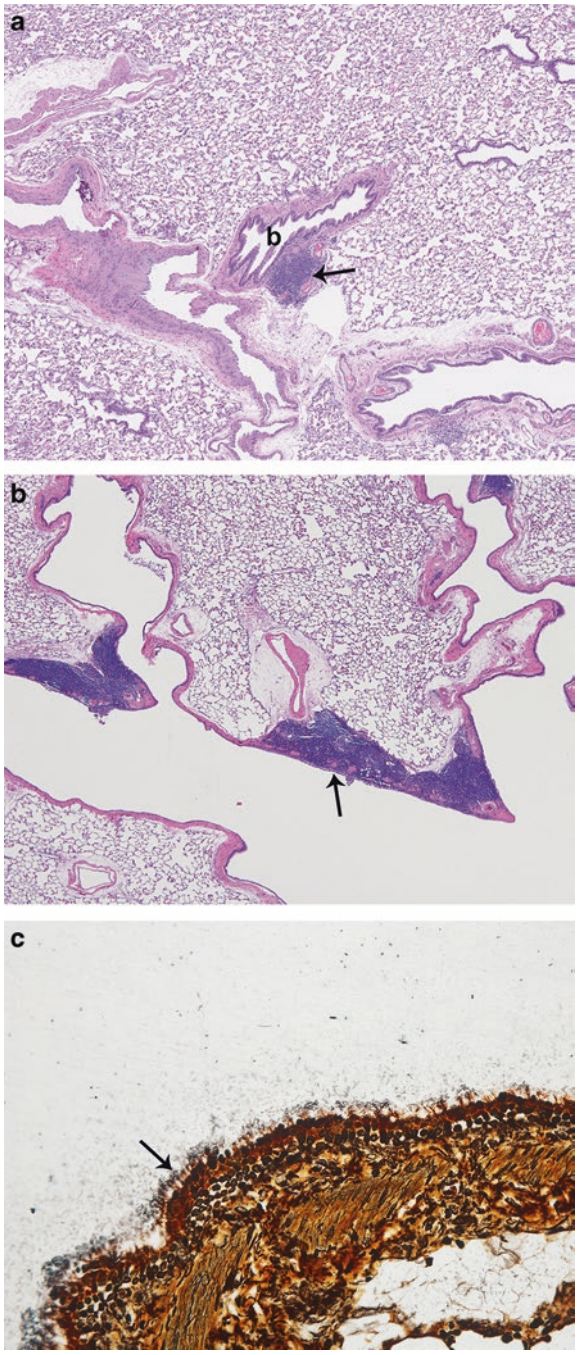

Fig. 7.10 (**a**) The lung of a non-dosed Sprague-Dawley rat has an accumulation of bronchus-associated lymphoid tissue (BALT) (*arrow*) located adjacent to a bronchiole (*b*). This degree of BALT expression, which is constitutive in rats, would be considered within the range of normal. H&E stain, 4× objective magnification. (**b**) The lung of a rat with confirmed CAR bacillus infection has increased prominence of bronchus-associated lymphoid tissue (BALT) (*arrow*). H&E stain, 4× objective magnification. (**c**) A The Warthin-Starry silver stain performed on lung tissue shows a myriad of silver-positive (*black*) organisms (*arrow*) within the ciliary border of bronchial epithelial cells. Warthin–Starry silver stain, 40× objective magnification

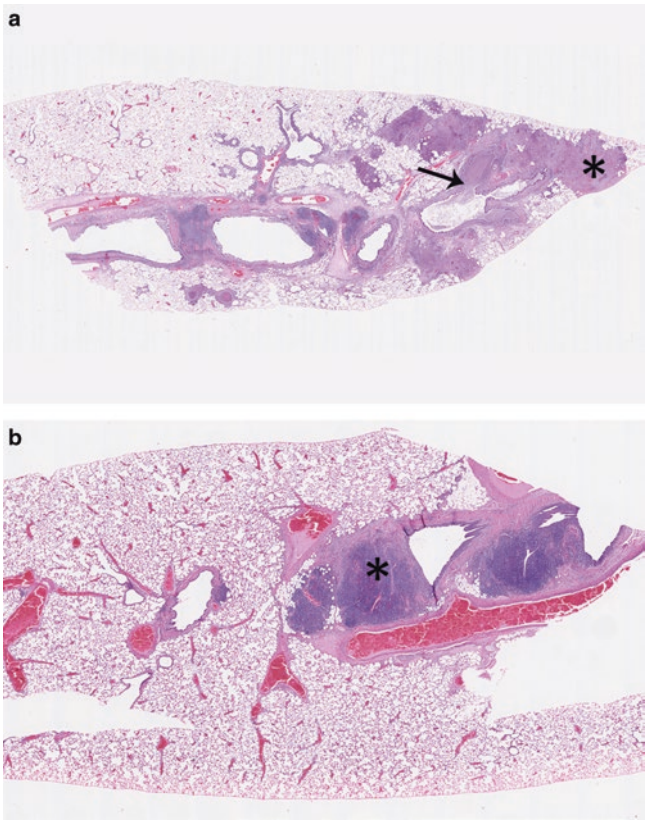

Fig. 7.11 (**a**) The lung lobe from a laboratory rat with chronic respiratory disease (CRD), consistent with chronic murine mycoplasmosis. Note the multifocally intense inflammatory cell infiltrations (*) and bronchioles (*arrow*) plugged with inflammatory cells and tissue debris. H&E stain, 0.68 objective magnification. (**b**) A morphologically different presentation of chronic respiratory disease in a laboratory rat which is manifested primarily by expansion of bronchus-associated lymphoid tissue (BALT) (*). The BALT expansions may be of striking magnitude, and may resemble neoplastic lymphoid proliferation. H&E stain, 1.43× objective magnification

7.5.8 Reactivated Viral Infections in Nonhuman Primates

Cynomolgus macaques (*Macaca fascicularis*) are widely used in nonclinical toxicology testing (Ambagala et al. 2011), and various nonhuman primate species, primarily rhesus macaques (*Macaca mulatta*), cynomolgus macaques, and baboons (*Papio* spp.) have been used extensively in solid organ transplant research (Haustein et al. 2008). A number of viral pathogens affect these animals (Haley 2012), and some of the pathogenic viruses normally exist in a cryptic status. Cynomolgus macaques may harbor a polyomavirus that remains cryptic unless

the animals are immunosuppressed, when the released viral infection causes tubulointerstitial nephritis, apoptosis of tubular epithelial cells, and progressive destruction of collecting ducts in the kidney (van Gorder et al. 1999). There is some evidence that polyomavirus infection in immunosuppressed cynomolgus macaques also results in intestinal lesions (van Gorder et al. 1999). A study of the seroprevalence of polyomavirus infection in various species of macaques revealed high seropositive rates in cynomolgus macaques, especially in animals imported from China (Verschoor et al. 2008). Notably low polyomavirus seropositivity rates (8.8%) were detected in cynomolgus macaques from Mauritius. Viral inclusion bodies are present in the kidney during the acute phase of the viral infection (Fig. 7.12b, c), but the hallmark inclusion bodies may not be present in subacute or chronic lesions (van Gorder et al. 1999).

Though not directly related to non-clinical toxicology studies and drug development, lessons learned in viral pathology and organ transplant pathology have applicability in toxicology studies. For example, reactivated cytomegalovirus infection was the most common opportunistic infection in rhesus macaques that were experimentally infected with the HIV/Delta lentivirus (Baskin 1987). A cytomegalovirus (CyCMV) with many similarities to the rhesus cytomegalovirus (RhCMV) has been characterized in cynomolgus macaques (Ambagala et al. 2011; Marsh et al. 2011). In a review of the effect of endogenous virus reactivation on xenotransplantation research, it was estimated that 14% of recipient nonhuman primates had infections due to reactivation of viruses such as cytomegalovirus, polyomavirus and Epstein-Barr virus (Haustein et al. 2008). Serological analysis of postmortem sera and tissues from 45 immunosuppressed and xenografted cynomolgus macaques showed 100% seropositivity for cytomegalovirus (Cavicchioli et al. 2015). The CyCMV down-regulates expression of MHC Class I on infected cells and has specific effects on humoral and cell-mediated immunity (Marsh et al. 2011), thus may result in complications beyond the virus-associated lesions in various organs. Reactivated cryptic cytomegalovirus infection is a common cause of serious intestinal lesions in immunosuppressed humans such as organ transplant patients (Haustein et al. 2008; Jonker et al. 2004; Pearson et al. 2002), therefore this possibility should be considered if non-clinical toxicology studies in nonhuman primates have unexplained intestinal complications. Routine histopathologic examination has been found to be somewhat unreliable in the detection of cytomegalovirus infection in humans, but immunohistochemical or in-situ hybridization staining provides more reliable indication of the viral infection (Muir et al. 1998; Mills et al. 2013).

Given the common occurrence of on-target or off-target immunosuppression associated with xenobiotic administration, the pathologist should give careful consideration to the possibility of activated cryptic viral infections when inflammatory lesions are observed, particularly in cynomolgus monkeys. While these infections are an indirect effect of test article, they do not indicate a direct toxic effect on the organ or tissue that expresses the viral infection.

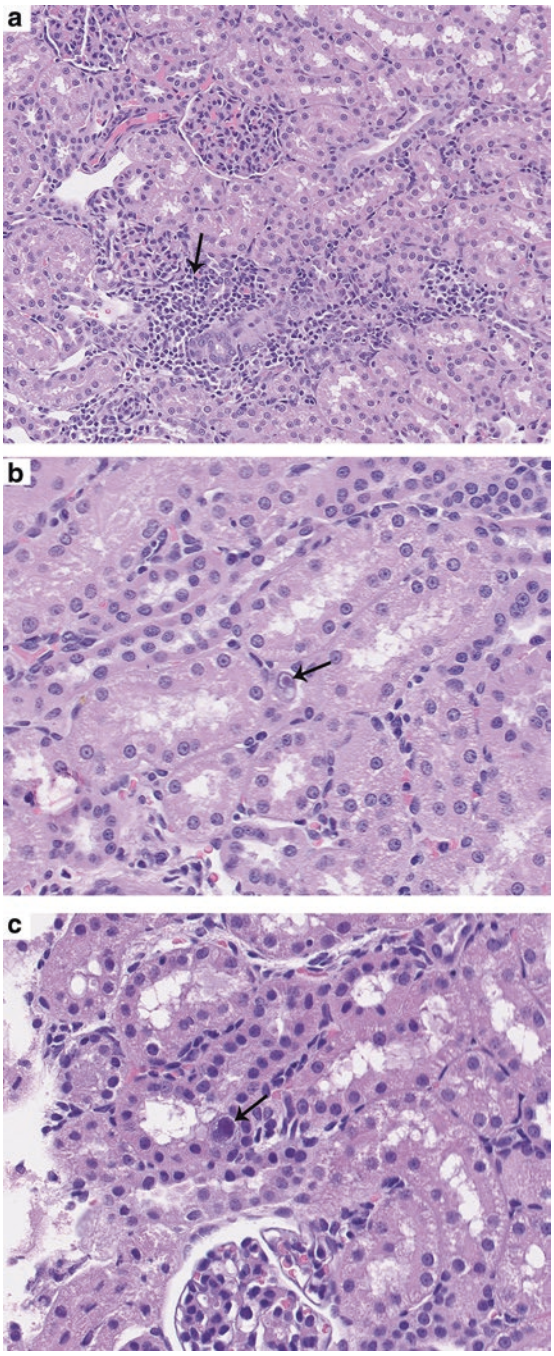

Fig. 7.12 (**a**) The renal cortex of a cynomolgus macaque has a moderate infiltration of mononuclear inflammatory cells (*arrow*), indicating a subacute inflammatory lesion. The renal lesion was an incidental finding, unrelated to experimental manipulation. H&E stain, 10× objective magnification. (**b**) Renal tubular epithelial cells of the cynomolgus macaque shown in Fig. 7.12 had a small number of intranuclear inclusion bodes (*arrow*). Cynomolgus macaques are known to have cryptic polyomavirus and cytomegalovirus infections which may become clinically overt due to

7.5.9 Candidiasis as a Complication of Immunomodulation

Candida albicans is a commensal organism that is commonly found in the oral cavity and skin of many mammalian species, including humans (Brown et al. 2012). Resistance to Candida infection involves cooperation between the adaptive and innate immune systems, the latter based on recognition of pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-1-like receptors (RLTs) (Netea et al. 2015). Signaling by CLRs following recognition of β-1,3-glucan and β-1,6-glucan in the fungal wall is of primary importance in innate defense against *Candida* spp. These glucans are typically shielded from immune surveillance by mannoproteins on the surface of the organisms, but the glucans become exposed on budding yeasts and the hyphal (mycelial) forms of the organism (Davis et al. 2014). In immunocompetent individuals, adaptive cell mediated immunity to the fungus prevents overgrowth and symptomatic infection (Fidel and Sobel 1994). The organism becomes an opportunistic pathogen when host homeostasis is disrupted, e.g. with neutropenia of diverse causes or following treatment with corticosteroids or antibiotics (Netea et al. 2015). Neutropenia serves as a predisposing factor, but alteration of cell-mediated adaptive immunity is necessary for establishment of clinically relevant disease (Romani 2000). An important virulence factor is the switch from a pure yeast form to a mixed yeast and filamentous (mycelial) form (Kobayashi and Cutler 1998). It is suspected that the mycelial form allows the organism to penetrate tissues and evade the adaptive immune system, but the yeast form is the proliferative form in infected tissues (Romani 2000). See Netea et al. for a review of immune defense against Candida (Netea et al. 2015).

Pathological candidiasis is encountered with some frequency in non-clinical toxicology studies, particularly studies that involve a combination of generalized lymphoid suppression and bone marrow hypocellarity with reduction in neutrophilic precursors (myeloid suppression) (Fig. 7.13). The least pronounced, and presumably earliest, manifestation of candidiasis is presence of fungal spores in the superficial keratin layer of the tongue, oropharynx, esophagus and cardiac region of the stomach (Fig. 7.14a). Presence of mycelial forms suggests the potential for increased virulence of the organisms. At this stage, there typically is no host reaction in the form of tissue alteration or inflammatory cell infiltration. More advanced fungal lesions involve erosion or overt ulceration of mucosal surfaces, with associated inflammatory cell infiltration (Fig. 7.14b, c). The lesions may involve the tongue, pharynx, esophagus and nonglandular region of the stomach of rodents, as

Fig. 7.12 (continued) stress or pharmacological immunosuppression. The exact identity of the virus(s) involved in this animal was not determined, but variation in the histologic appearance of the inclusion bodies in the kidney suggested both cytomegalovirus and polyomavirus infection. The 'owl's eye-type' inclusion body shown in this image suggests cytomegalovirus infection. H&E stain, 40× objective magnification. (c) Other intranuclear inclusion bodies noted in the animal presented in Fig. 7.12a, b were larger and essentially filled enlarged nuclei, suggesting polyomavirus infection. H&E stain, 40× objective magnification

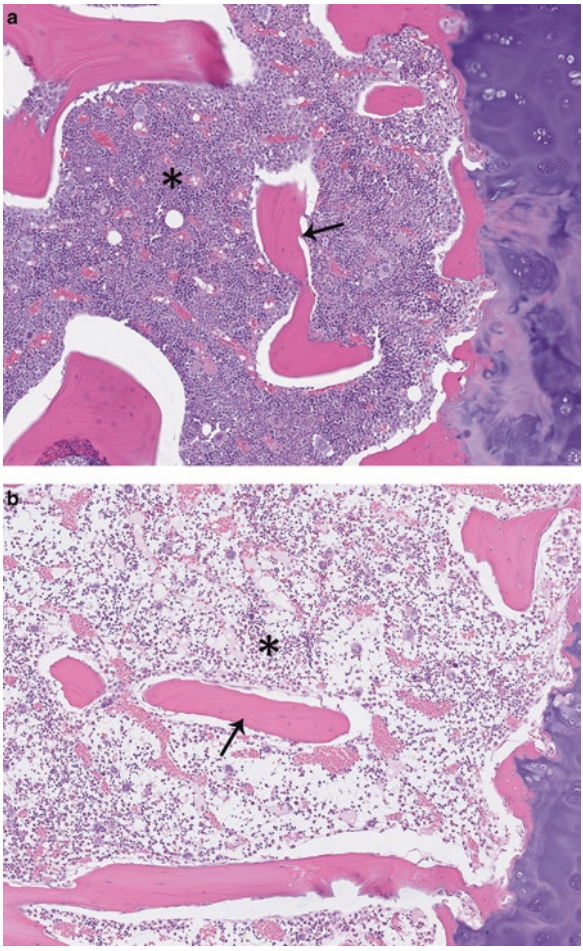

Fig. 7.13 (**a**) Sternal bone marrow from a non-dosed, young adult cynomolgus macaque. Note the dense population of hematopoietic cells (*) surrounding osseous spicules (*arrow*) of the marrow cavity. H&E stain, 10× objective magnification. (**b**) Sternal marrow of a young adult cynomolgus macaque dosed with a test article that had immunosuppressive and bone marrow suppressive activity. Note the reduced population of hematopoietic cells (*) surrounding osseous spicules (*arrow*). H&E stain, 10× objective magnification. (**c**) Sternum with bone marrow from a young Sprague Dawley rat. Note the intense hematopoietic cell population in the marrow cavity (*). H&E stain, 1.25× objective magnification. (**d**) Sternum with bone marrow from a young Sprague-Dawley rat that was dosed with a test article that had known (pharmacologically desirable) immunosuppressive activity. Note the hematopoietic cell population in the central aspect of each sternebral marrow cavity is partially replaced by clear spaces, the latter representing adipocytes. H&E stain, 1.25× objective magnification

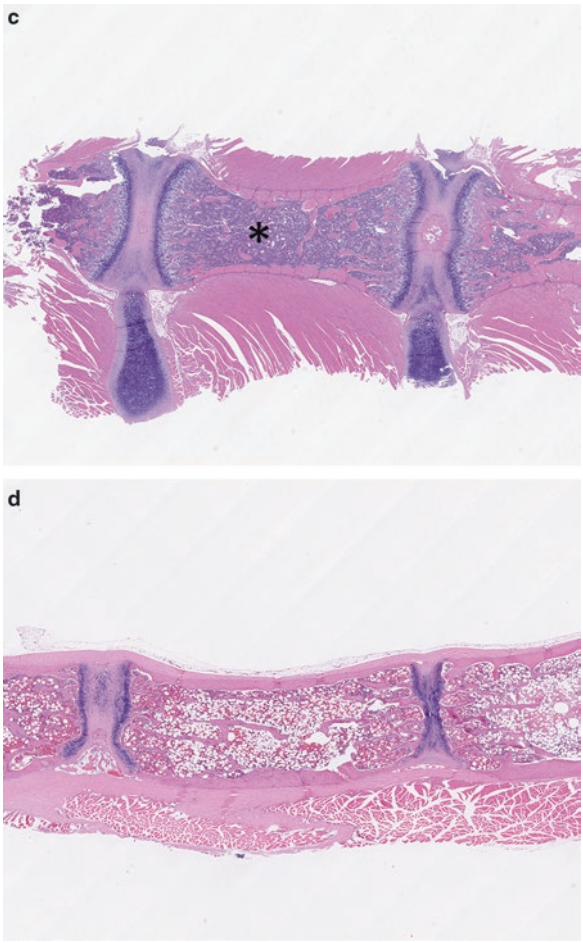

Fig. 7.13 (continued)

well as the nonglandular orifice of the gastric cardia in nonhuman primates. It is imperative that histologic sections should include the esophageal-gastric junction of the gastric cardia, as this is a common site of localization. Advanced cases of candidiasis may include dissemination of fungal organisms to internal organs such as kidney and liver (Fig. 7.14d, e), where massive fungal colonies are associated with tissue necrosis and inflammation. Particularly careful histologic examination of the disseminated lesions is indicated, as concurrent infection by other fungal or bacterial organisms may be present.

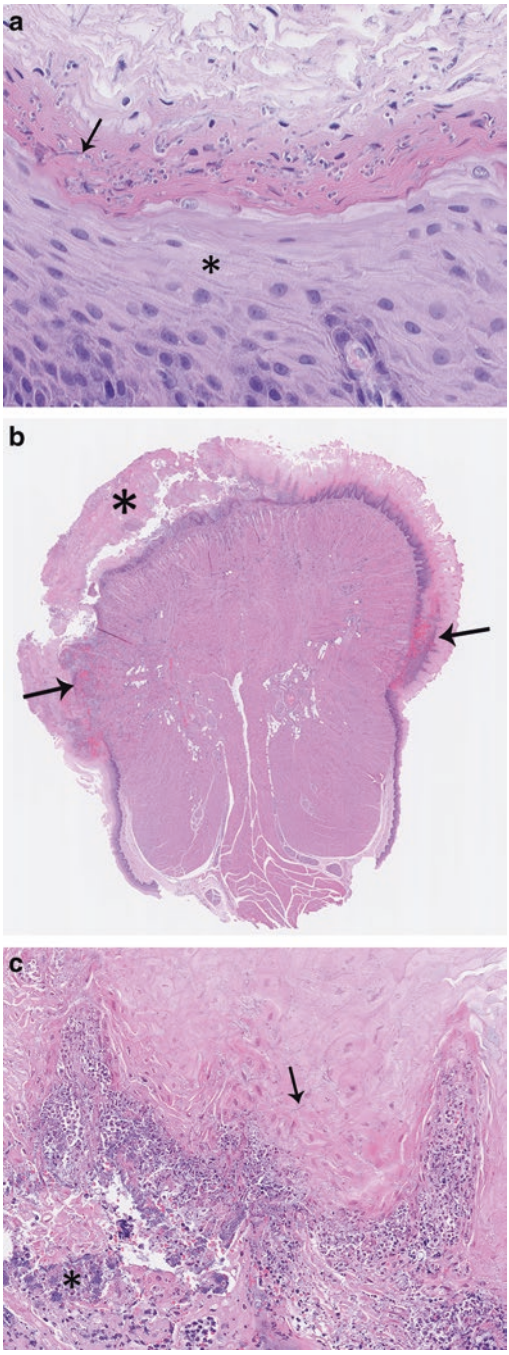

Fig. 7.14 (**a**) The esophagus of a cynomolgus macaque has numerous spores and short mycelial forms (*arrow*) that are consistent with *Candida albicans*. Note the organisms are contained with the superficial keratin layer, with no penetration into the underlying viable keratinocytes (*). Also note the absence of inflammatory cell infiltration into the viable keratinocyte layer. Similar organisms were present in the superficial keratin layer of the tongue and gastric cardia. The animal was

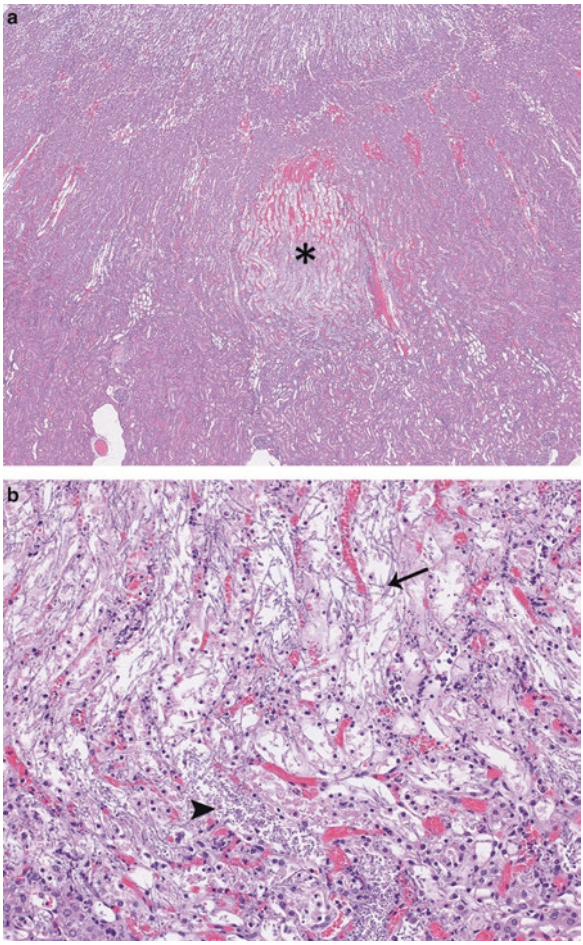

Fig. 7.14 (continued) dosed with a candidate antimicrobial agent, which would be expected to alter the intestinal microbiome. See narrative text for details of the relationship between the intestinal microbiome and *Candida* overgrowth. H&E stain, 40× objective magnification. (**b**) Cross-section of the tongue of a Sprague-Dawley rat that was dosed with a known (pharmacologically desirable) immunosuppressive agent. Note the areas of hemorrhage and inflammatory cell infiltration (*arrows*) and thick crust (*) composed of inflammatory cells and tissue debris. Multiple animals of the high-dose treatment group had similar lesions in the pharynx, esophagus and non-glandular region of the stomach. H&E stain, 1.25× objective magnification. (**c**) A higher magnification of the lesion shown in Fig. 7.14b has numerous spores and mycelial forms (*arrow*) associated with the ulcerated region of the tongue. Deeply basophilic bacterial colonies (*) represent secondary bacterial infection. H&E stain, 20× objective magnification. (**d**) The kidney of a rat from the high-dose group of the study represented in Fig. 7.14b, c has an area (*) of necrosis, vascular congestion and hemorrhage. H&E stain, 2.5× objective magnification. (**e**) Higher magnification of the lesion shown in Fig. 7.14d shows numerous spores (*arrowhead*) and mycelial forms (*arrow*) that are consistent with *Candida albicans*. The non-glandular region of the stomach (not shown) had a pronounced ulcerative lesion with numerous Candida-type organisms, which was presumed to be the source of the organisms seen in the kidney. H&E stain, 20× objective magnification

7.5.10 Demodex-Related Skin Lesions

Demodex mites colonized the hair follicles and sebaceous glands of mammals millions of years ago and have remained relatively unchanged in this protected ecologic niche since then (Ferrer et al. 2014). Numerous species of *Demodex* have been described, with species names typically referring to the specific parasitized host. The host immune system detects and tolerates presence of the mites, though toll-like receptor-2 (TLR-2) has been shown to recognize chitin in the mite exoskeleton and to elicit an innate immune response (Ferrer et al. 2014). Establishment and progression of the clinical disease in dogs is influenced by numerous factors including genetic defects, alteration of skin's structure and biochemistry, immunological disorders, hormonal status, breed, age, nutritional status, oxidative stress, length of hair coat, stage of estrus cycle, parturition, endoparasitism and debilitating diseases (Singh and Dimri 2014). Of these, the immune status is thought to be the most significant. The adaptive immune response to mites is complex, involving both cellular and humoral elements and requiring the involvement of the CD28 co-stimulatory molecule (Ferrer et al. 2014).

Though clinical disease attributable to *Demodex canis* is rare in the purpose-bred dogs typically used in non-clinical toxicology studies, the mites are occasionally encountered as incidental findings in histologic sections of skin (Fig. 7.15).

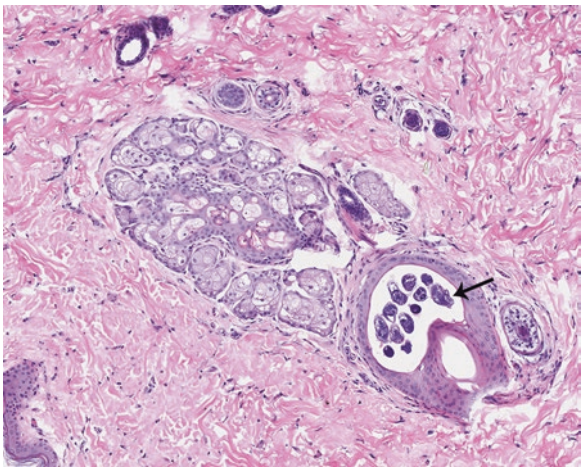

Fig. 7.15 A hair follicle that was fortuitously included in a routine section of skin from a young beagle dog contains multiple cross-sections of mites (*arrow*), consistent with *Demodex* spp. Note the absence of associated inflammatory cell infiltration. It is uncommon to encounter histologically intact mites in skin sections of dogs from non-clinical toxicology studies, but these studies sometimes have a low incidence of nonspecific perifollicular dermatitis that is consistent with *Demodex*-associated inflammation. Depending on the dose-related incidence pattern of these lesions, they may be interpreted as an indication of test article-related immunomodulation. The mite infestation shown here was seen in a dog of the lowest dosage group, and there were no similar lesions in the two higher dosage groups, thus the mite infestation was considered to be an incidental finding. H&E stain, 10× objective magnification

The mite infestations may or may not be associated with inflammatory cell infiltration and/or degenerative changes in the hair follicles and sebaceous glands. A more common occurrence is the presence of perifollicular inflammatory lesions in the skin ('perifolliculitis') with no discernible mites in the histologic sections. Given the known involvement of immunologic deficits in the establishment and progression of demodicosis, it is reasonable to view any evidence of demodicosis or the associated perifolliculitis with some suspicion, particularly if there is additional evidence of immunomodulation in the study. Regulatory reviewers may group these cutaneous lesions together with nonspecific inflammatory lesions in other organs to raise concerns regarding generalized immunomodulation associated with xenobiotic administration. In order that interpretations and conclusions may be accurate, the pathologist should provide details regarding the histologic features and suggested pathogenesis of inflammatory lesions, including those suspected to be associated with *Demodex* spp.

Though not as well-known as demodicosis in dogs, demodicosis has been reported in a number of nonhuman primate species, including rhesus macaques (*Macaca mulatta*) (Karjala et al. 2005; Starost et al. 2005). In rhesus macaques the mite infestation is most pronounced in the perianal skin. In a prospective survey based on necropsy of 53 rhesus macaques, 19 were found to have *Demodex* mites (Starost et al. 2005). There was no difference in the incidence of *Demodex* infestation in immunocompromised versus immunocompetent animals, but the associated inflammatory lesions were more pronounced in immunocompromised animals (Starost et al. 2005).

7.5.11 *Oesophagostomiasis in Nonhuman Primates*

Parasitic granulomas associated with *Oesophagostomum* spp. nematodes were commonly encountered in the large intestine of wild-caught nonhuman primates (Abbott and Majeed 1984), but are much less common in the captive-bred animals used in modern non-clinical toxicology studies. However, the typical necrotizing granulomatous are occasionally encountered. The incidence and severity of *Oesophagostomum*-related lesions were increased in a study of a known immunosuppressive drug (Chellman et al. 1992), therefore, presence of the parasite-related lesions raises concerns with regard to xenobiotic-related immunomodulation.

7.5.12 *Pneumonyssus Mites and Lung Pigment in Nonhuman Primates*

Bronchitis and bronchiolitis in the lungs may be caused by *Pneumonyssus semicola* mite infestation, which is commonly encountered in wild-caught cynomolgus and rhesus monkeys as well as baboons. Intact parasites are uncommonly included

in histologic sections, but the mite infestation is revealed by presence of the characteristic pigment contained within alveolar macrophages (Abbott and Majeed 1984). Mite-associated pulmonary pigment must be differentiated from the anthracosilicotic pigment accumulation that commonly results from inhalation of particulate-laden air in urban environments.

7.5.13 Cercopithecine Herpesvirus Type-1 (Herpes B virus) in Nonhuman Primates

Infection of nonhuman primates with *Cercopithecine herpesvirus type-1* (Herpes B virus) represents a critical development in non-clinical toxicology studies due to the potential for transmission of fatal infections to humans. B virus infection in susceptible macaques typically results in a mild clinical presentation that includes oral vesicles and ulcers, and may include similar lesions on the skin and conjunctiva (Hunt et al. 1978). The cynomolgus (*Macaca fasciularis*) and rhesus (*M. mulatta*) monkeys that are commonly used in non-clinical toxicology studies are among the macaque species that are susceptible to the infection. There is evidence that this viral infection may persist in captive-bred nonhuman primate colonies. Serologically positive specimens were detected in 45% of rhesus monkeys in a Brazilian colony that had been closed to new animal introductions for more than 70 years (Andrade et al. 2003). There are reports of clinical exacerbation of herpes B virus infection in immunosuppressed nonhuman primates. After three months of dosing with the highest dose of an immunosuppressive test article, three of 14 cynomolgus monkeys developed oral lesions that were consistent with B virus infection (Chellman et al. 1992). This necessitated early removal of all high-dose animals from the study due to public health concerns related to herpes B virus infection. A young female rhesus monkey infected with simian immunodeficiency virus had an oral ulcer that contained herpetic inclusion bodies, and subsequent testing indicated the presence of herpes B virus (Bailey and Miller 2012). Any evidence of ulcers or inflammatory lesions in the oral cavity, skin or conjunctiva of susceptible nonhuman primate species should be viewed with great concern, and immediate steps taken to protect exposed humans from this potentially lethal human pathogen.

7.5.14 Atypical Infections

Increased incidence and/or severity of traditional pathogens of the various laboratory animal species is the more common as a manifestation of immunosuppression, but infections by pathogens that are unusual for the species should also raise the level of suspicion. For example, infection by *Lawsonia intracellularis*, a common disease of domestic swine, causes the hallmark microscopic change of

inflammation and enterocyte proliferation in the ileum (Vannucci and Gebhart 2014). Sporadic cases have been reported in other species, primarily nonhuman primates (Klein et al. 1999; Lafortune et al. 2004; Wamsley et al. 2005). Lawsonia infection is rarely reported in dogs (Husnik et al. 2003; Lawson and Gebhart 2000; Cooper and Gebhart 1998). In the author's (GAP) experience, the occurrence of microscopically typical proliferative lesions in the ileum of multiple dogs of a non-clinical toxicology study, including one dog from the vehicle control group, caused considerable concern on the part of the responsible scientists. Warthin Starry silver stains revealed the characteristic organisms in enterocytes of the ileum, and immunohistochemical staining indicated the organisms were *Lawsonia intracellularis*. Careful review of the animal histories revealed no evidence of contact with swine, either in the toxicology laboratory or the dog production facility.

7.5.15 Non-specific Inflammatory Lesions

Given that a major function of the immune system is protection of the host from invasion by pathogens, histologic evidence of inflammation in any tissue raises concerns that xenobiotic-related immunomodulation has allowed pathogens to breach the protective barrier. While it is typically impossible to detect definitive evidence of a specific etiology for these inflammatory lesions in individual organs and animals, it is important for the pathologist to provide whatever interpretation is possible with regard to the pathogenesis of the lesions. Citing pertinent literature showing the common or uncommon prevalence of the lesions helps readers reach accurate conclusions regarding the importance of the histologic observations. Any atypical histologic features of the lesions should be noted (Fig. 7.16). Conversely, a definitive indication that the observed lesions were typical of spontaneous ('background') findings is helpful to readers as they make decisions regarding the importance of specific histologic observations.

The liver commonly has clusters of individualized infiltrative cells that are superficially consistent with inflammatory cells. Experience suggests these cellular populations may be divided into three categories: (1) individualized lymphocytes that tend to be widely dispersed along biliary tracts, (2) clusters of lymphocytes and macrophages that are sometimes associated with degenerated hepatocytes, and (3) clusters of hematopoietic cells. The first category of cells is most likely a component of the diffuse mucosal immune system of the intestine, which also serves in extrathymic T cell generation following involution of the thymus (Abo 2001, 1993). The second category of cells is consistent with the historically described 'bacterial showering' from the intestine, by which a small number of bacteria reach the liver via the portal circulation and provoke a localized inflammatory reaction. The third category, clusters of hematopoietic cells, is a remnant of the prominent hematopoiesis that occurs in the liver during embryogenesis, and has been shown to persist for several weeks after birth (Parker et al. 2015). It is important that the pathologist

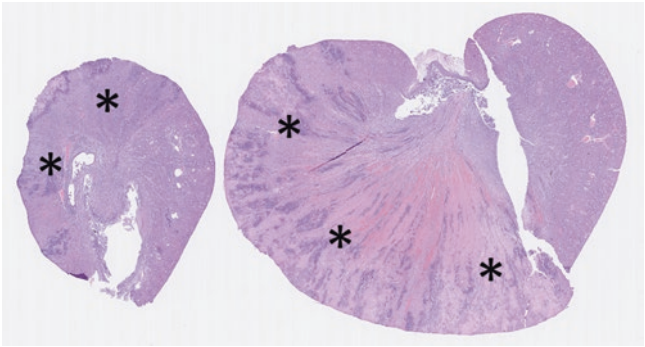

Fig. 7.16 Longitudinal (*right image*) and cross-sections (*left image*) of the kidney of a Sprague-Dawley rat have extensive areas (*) of necrosis, hemorrhage and inflammatory cell infiltration. Multiple deeply basophilic areas represent bacterial colonization. Ascending inflammatory lesions of the urinary tract are seen with some frequency in laboratory rats, and are presumed to be of bacterial origin. However, lesions of the severity demonstrated here are atypical. The subject rat was treated with a known immunosuppressive (pharmacologically desirable) test article, and the immunosuppression may have increased the severity of the spontaneous disease process. Increased incidence or severity of known background lesions should raise the level of suspicion for xenobiotic-related immunosuppression. H&E stain, 0.35× objective magnification

should recognize these different cell populations, even though they are superficially similar upon light microscopic examination. Increases or decreases in any of the cell populations may occur due to diverse pathogenic mechanisms. It is interesting to note that the peribiliary lymphocytic population in the liver tends to be diminished or absent in studies involving immunosuppression.

7.5.16 Amyloid Deposition in Tissues

Amyloid presents histologically as extracellular protein deposits that are selectively stained by Congo red stain, and exhibit green ('apple-green') birefringence when viewed by polarization microscopy. Amyloid deposits are seen with a number of disease processes, and often involve multiple organs. The specific protein constituents of amyloid vary depending on the disease process involved, but serum amyloid A protein is commonly involved. A number of diseases that involve amyloid deposition are based on immunological disorders, therefore, presence of amyloid deposition in tissues arouses suspicion of immunopathy. Amyloid is commonly deposited in renal glomeruli, in which location the deposits must be distinguished from immune complex depositions or glomerular hyalinosis (Hoane et al. 2016). Volume 2, Chap. 6 presents more extensive information regarding immune-mediated glomerular disease and its relationship with amyloidosis.

Systemic amyloidosis is a common cause of morbidity or mortality in CD-1 mice (Maita et al. 1988), which are commonly used in non-clinical toxicology studies.

Many organs may contain amyloid deposits, but the kidneys, small intestine, liver and thyroid glands are particularly common sites of involvement. This spontaneous disease of mice is so common and so well-known that special stains to confirm the nature of the tissue deposits typically are not required. In situations where confirmation is required, Congo red staining with demonstration of 'apple-green' birefringence upon examination via polarized light is the standard confirmation procedure. It should be noted that selective staining by Congo red in the absence of the green birefringence with polarization microscopy does not constitute a confirmatory test. If equivocal results are obtained with Congo red staining, it is sometimes helpful to perform the stains on histologic sections that are thicker (e.g. 10–12 μm) than standard sections.

Spontaneous, often idiopathic, amyloid deposition is seen with some frequency in many species, including dogs and nonhuman primates. Amyloidosis is uncommonly seen in laboratory rats, presumably due to the absence of serum amyloid A protein as an acute phase reactant in that species (Gruys and Snel 1994; Rossmann et al. 2014).

During histopathologic evaluations of non-clinical toxicology studies it is important for pathologists to detect the dose-related incidence of lesions, including amyloidosis. Histologic changes are typically recorded in the organs that harbor the lesions, and a simple computer-assisted tabulation clearly indicates dose associations. This process is more complicated with lesion complexes, such as amyloidosis, that involve multiple tissues. No single organ may consistently have amyloid deposition, thus the histopathology data tables do not indicate the dose-related incidence of animals affected by amyloidosis. This problem may be solved by recording such lesions under a category of 'systemic lesions' or other similar term. With this approach, if one animal has amyloid deposition only in the kidneys, another has amyloid only in the intestine, and a third animal has amyloid only in the thyroid gland, the tabulation of amyloidosis under 'systemic lesions' will give the true incidence of three occurrences of amyloidosis. If this approach is used, the amyloid depositions in individual tissues should also be recorded to allow detection of any treatment-related alteration in the incidence or severity of amyloid deposition in individual tissues.

7.5.17 Pseudoamyloid

An eosinophilic substance (ES) is commonly observed in the mouse nasal septum and increases in volume with aging (Doi et al. 2007; Haines et al. 2001). This material has been described as amyloid, however, this identification was questioned because the nasal deposits did not have a positive reaction to Congo red staining. Subsequent studies have shown the material to be a non-amyloid substance that is probably produced by the nasal gland epithelial cells (Doi et al. 2007). It is important that pathologists recognize this material as a separate entity from amyloidosis, otherwise the true incidence of amyloidosis in a study may be distorted by the presence of pseudoamyloid in the nasal cavity.

7.5.18 Miscellaneous Histologic Changes

A number of incidental histologic findings have potential to cause consternation with regard to potential immunotoxic effects in toxicology studies. Some of the more common observations are summarized below.

Heterotopic (ectopic) splenic tissue in the abdominal cavity is encountered with some frequency in various animal species. As presented in Volume 1, Chap. 4, the spleen develops from multiple independent primordia that subsequently fuse to form a single organ. On occasion those separate structures fail to completely fuse, resulting in one or more splenic heterotopia. In addition, traumatic injury to the abdominal cavity can rupture the spleen. If the animal survives this injury, viable fragments of splenic tissue may be present in the abdominal cavity. This latter situation is most likely to occur in dogs that are kept as pets, which have an unfortunately high rate of encounter with moving automobiles, but is possible following injury to the abdominal cavity of a laboratory animal. It should be noted that rabbits have an exceptionally high incidence of heterotopic spleen, to the degree that some laboratories do not record them as gross necropsy observations. There typically is no evidence of traumatic injury to the rabbits, suggesting this is a developmental defect in that species.

Lymphoid follicles in the spleen and lymph nodes of nonhuman primates commonly have deposits of homogeneous eosinophilic material (Fig. 7.17) that are

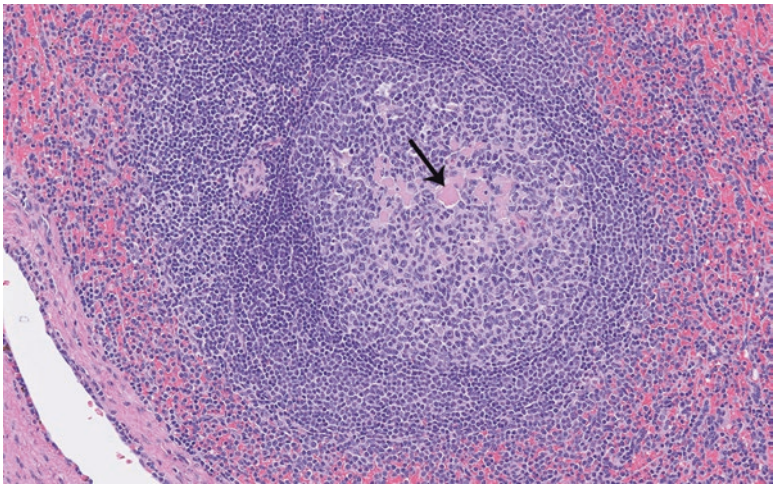

Fig. 7.17 A secondary lymphoid follicle in the spleen of a cynomolgus macaque has accumulations (*arrow*) of eosinophilic material that is of uncertain genesis and significance. The material is histologically similar to amyloid, but special histochemical stains (e.g., Congo red staining) indicate it is not amyloid. Similar material is sometimes seen in lymphoid follicles of lymph nodes of non-human primates. It is important for the histopathologist to recognize this material as a normal species-specific histologic feature rather than an indication of a pathologic process. H&E stain, 20× objective magnification

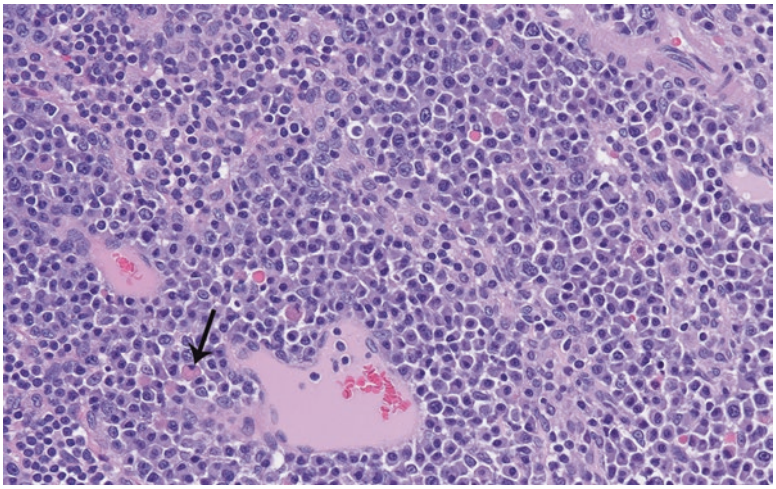

Fig. 7.18 The mandibular lymph nodes of young rats commonly have massive accumulations of plasma cells in the medullary cords. Plasma cells in this image are identified by eccentrically placed nuclei and a moderate amount of amphophilic cytoplasm with a paranuclear eosinophilic zone that represents the Golgi apparatus. Mandibular lymph nodes with plasmacytosis commonly contain Mott cells (*arrow*), which are thought to be plasma cells that are engorged with secretory product. Affected lymph nodes may be noted as grossly enlarged at necropsy. H&E stain, 40× objective magnification

superficially consistent with amyloid deposition upon initial microscopic examination, but special staining procedures indicate the material is not amyloid.

Mandibular lymph nodes of young rats commonly have pronounced accumulations of mature plasma cells within medullary cords (Fig. 7.18). Such prominent plasmacytic accumulations are not typically seen in other lymph nodes of rats, nor in mandibular lymph nodes of species other than rats. The cause of the plasmacytosis is unknown. The histologic change is nearly ubiquitous, and is of no known significance. However, in some animals the plasmacytosis may be so pronounced as to result in a grossly visible enlargement of one or both mandibular lymph nodes, resulting in gross necropsy observations which must be correlated with histologic findings in typical practice. If plasmacytosis is recorded in the histopathology data only in the grossly altered lymph nodes, and the common occurrence of plasmacytosis is not routinely recorded, then the histopathology data tables will reveal only the incidence of the plasmacytosis that was sufficiently pronounced to result in gross necropsy observations. The gross necropsy observations are based on the subjective appraisal of the necropsy prosector, which then tracks forward to the tabulated incidence of histopathologic alterations. Should an excessive number those animals fall into the high-dose group, then an apparent dose-related incidence of histologically confirmed plasmacytosis in mandibular lymph nodes may be created when, in reality, the only change was a slight increase in the severity of the plasmacytosis in some rats, or a difference in the subjective appraisal of necropsy prosectors. Discovery of this apparent dose-related alteration in mandibular lymph nodes as the histopathology data tables are reviewed may necessitate a second

review of all mandibular lymph nodes in the study in order to record and grade the level of plasmacytosis in all animals. In order to avoid this effort, it is desirable to (a) record and grade the degree of plasmacytosis in mandibular lymph nodes of all rats as part of the initial histopathological evaluation or (b) meticulously record all occurrences of pronounced plasmacytosis, regardless of the presence or absence of a gross necropsy observation in the mandibular lymph nodes.

Intestinal lymphatics of various laboratory animal species sometimes are distended with well differentiated lymphocytes. Affected animals typically have no evidence of a lymphoproliferative disease in the immune system organs, and no histologic evidence of an inflammatory reaction that would explain the regional lymphocytosis. There is no histologic evidence of an associated deleterious effect on the intestinal tissues. While the exact nature of the histologic finding is uncertain, it can assume spurious importance in a non-clinical toxicology study if the study pathologist is unaware this is a 'background' finding and attaches toxicological significance to its presence.

7.6 'Whole Animal' Approach to Immunopathology

Histopathologic detection of immune system alterations should be based on the overall evaluation of immune system organs and related data, rather than focused on details of histologic changes in a single organ. Immune system alterations are typically manifested in multiple immune system organs, particularly the thymus, spleen and lymph nodes (Fig. 7.19a–f), but inconsistent histologic presentations in the various immune system organs are common. In addition to data entries to record the compartmental changes for each immune system organ, a summary entry regarding the pathologist's subjective estimation of the severity of immunomodulation in each individual immune system organ should be recorded at the time each animal is examined. It may also be helpful to make a data entry to summarize the overall histological changes in the animal at the time the microscopic examination is completed for each animal. Tabulation and review of the summary entries for the individual organs and whole animals may allow a more comprehensible view of any test article-related effects.

Investigation of aspects of the study other than the protocol-specified data may be necessary to complete the analysis of suspected immunomodulatory effects. For example, in one (unpublished) preclinical safety study (GAP) the rats had an apparent treatment-related incidence of cutaneous ulceration and bacterial infection which was commonly localized in the ventral neck region. The localization of the skin lesions was explained by observation of the remaining rats being held for the recovery phase of the study, which revealed the animals commonly had low-grade irritation of the ventral neck as a result of inserting the head into feed tubs. It was concluded that xenobiotic-related, and therapeutically desirable, immunomodulation allowed expansion of the husbandry-related minor skin irritation. Rats of the same study sometimes had unilateral enlargement of mandibular lymph nodes, consistent with 'reactive lymph nodes'

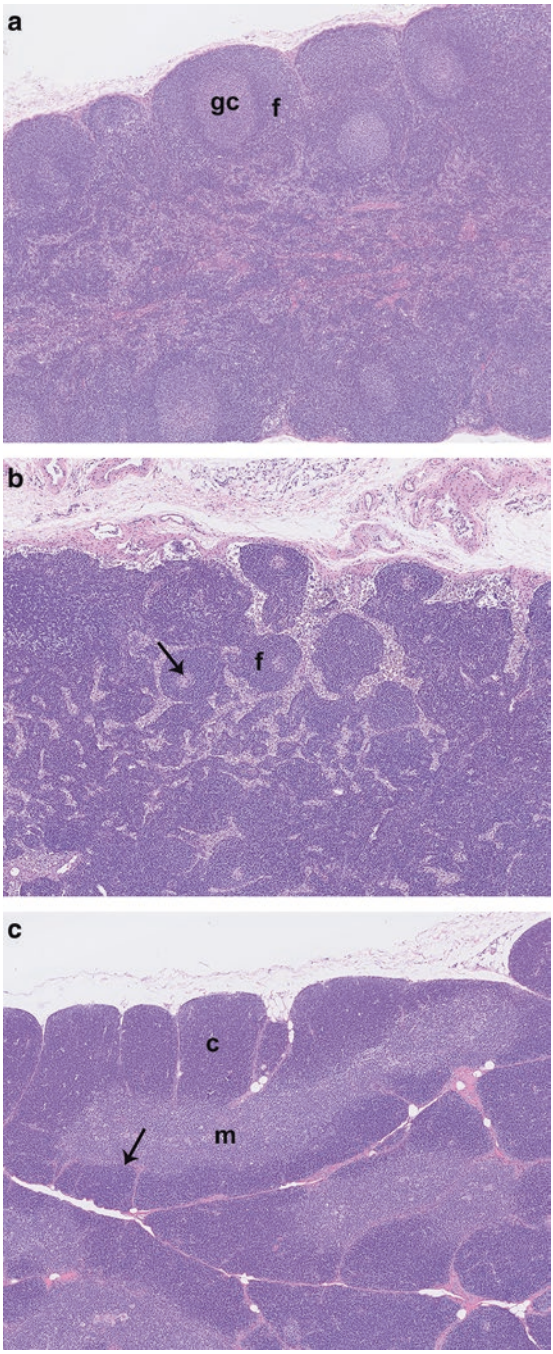

Fig. 7.19 (**a**) The mesenteric lymph node of a non-dosed cynomolgus macaque is histologically normal, with numerous follicles (*f*) and germinal centers (*gc*). H&E stain, 50× objective magnification. (**b**) The mesenteric lymph node of a cynomolgus macaque that was treated with a candidate

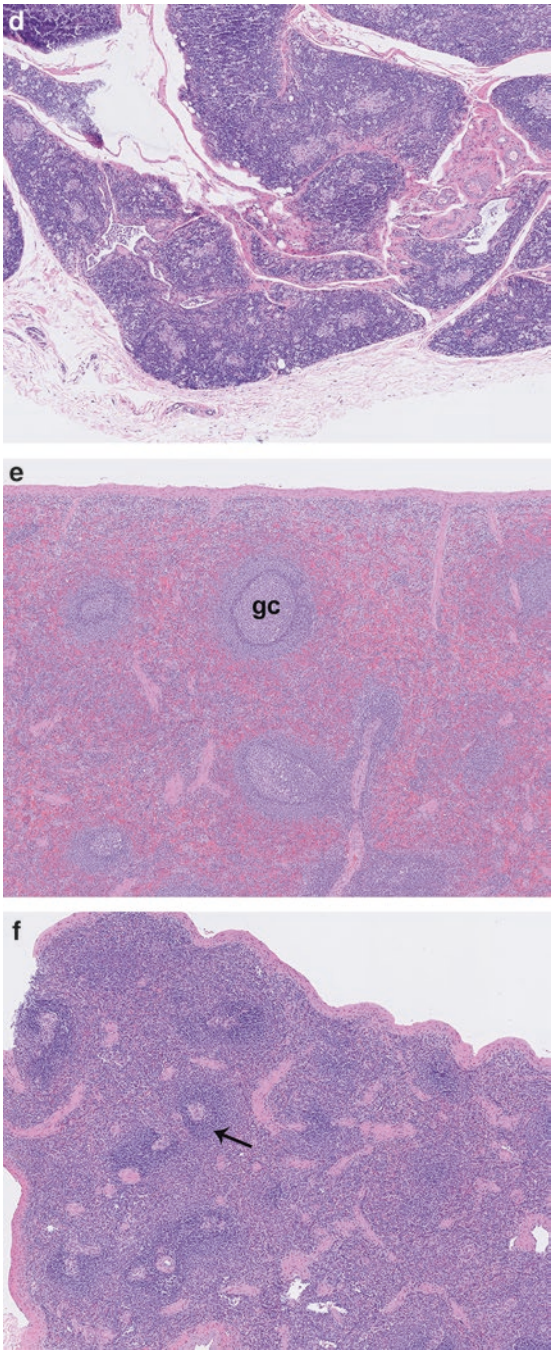

Fig. 7.19 (continued) drug has follicles (*f*) that are reduced in size. Sites of previous germinal centers (*arrow*) consist of an aggregation of cellular debris. Active follicles with germinal centers are not present. H&E stain, 5× objective magnification. (**c**) The thymus of a non-dosed cynomolgus

associated with the cervical skin ulceration, but the affected lymph nodes had a notable lack of the active secondary follicles with germinal centers that are typically present in reactive lymph nodes. Investigation of the mode of action of the test article revealed it would be expected to interrupt the germinal center reaction, thus absence of this feature in the histologic presentation was an expected manifestation of the test article's pharmacologic action rather than an indication of immunotoxicity.

7.7 Summary Points

- The pathology evaluation for detection of xenobiotic-related effects on the immune system should include integration of findings on a 'whole animal' basis, to include in-life observations, terminal body and organ weight analysis, gross necropsy observations, clinical pathology data, and histopathologic observations.
- The pathology evaluation should be preceded by review of findings in previous studies and any information regarding known effects of the test article, class effects or structure-activity relationships.
- The basic pathology evaluation should be viewed as a screening test rather than a definitive test for immune system effects.
- In performing the routine pathology evaluation there should be a very low threshold of suspicion for immune system effects, and any suggestions of immunomodulation should prompt more intensive and specific investigative assays.
- Study pathologists should offer an opinion as to the adverse or non-adverse nature of immune system effects, but determinations of no-effect levels should be based on all study data and not just the pathology data.
- There are two-way interactions between immunomodulation and diseases due to viruses, bacteria, fungi, protozoa and metazoan parasites. Immunomodulation may promote the development of these diseases and, conversely, presence of diseases or parasitism may cause immunomodulation.
- Aspects of the experimental design other than xenobiotic administration may cause or influence immune system alterations.
- Many pharmaceutical products are intended to have targeted immunomodulatory effects, and these may have expected or unexpected downstream effects.

Fig. 7.19 (continued) macaque is histologically normal, with highly cellular cortex (*c*), less densely cellular medulla (*m*), and a distinct border (*arrow*) between cortex and medulla. H&E stain, 5x objective magnification. D The thymus of a cynomolgus macaque that was treated with a candidate drug has reduced overall cellularity, with a marked reduction in cellularity. H&E stain, 5× objective magnification. (**e**) The spleen of a non-dosed cynomolgus macaque is histologically normal, with multiple prominent follicles that contain germinal centers (*gc*). H&E stain, 5× objective magnification. (**f**) The spleen of a cynomolgus macaque that was treated with a candidate drug has reduced overall cellularity of the lymphoid component ('white pulp'). The T cell-dependent periarteriolar lymphoid sheaths (*arrow*) are present, but follicles with germinal centers are not apparent. H&E stain, 5× objective magnification

Acknowledgements Preparation of figures by Cynthia Swanson, M.S. is gratefully acknowledged.

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Chapter 8
Enhanced Immunohistopathology

Susan A. Elmore

Abstract Enhanced histopathology is a tool that the pathologist can use to provide a more comprehensive evaluation of the immune system lymphoid organs. It involves the individual evaluation of the various compartments and subcompartments of each lymphoid organ with respect to size, cellularity, and other abnormal cellular changes such as fibrosis and necrosis. It also involves the use of descriptive rather than interpretive terms to describe the cellular and architectural changes. The advantage of such a detailed and comprehensive evaluation is that it allows for an understanding of the changes that can occur with cell production and cell death as well as with cellular trafficking and recirculation following administration of immunomodulatory drugs and chemicals. Moreover, by identifying the specific compartments affected, one might be able to have an early indication of which cell population is affected and also provide clues on the possible mechanism of action of the test article. This methodology is used with short term studies in conjunction with gross changes, body weights, organ weights, clinical chemistry and hematological measurements and may be a part of a larger immunotoxicity protocol that includes traditional immune functional and nonfunctional tests.

Keywords Enhanced histopathology • Lymphoid organs • Immune system • Immunomodulation • Immunotoxicity • Cellular trafficking

8.1 Introduction and Historical Perspective

Histopathological examination of lymphoid tissues can be a very sensitive tool in the evaluation of immunomodulatory effects of xenobiotics. Careful and methodological evaluation of lymphoid tissues has become increasingly important over the years (Kuper et al. 2000, 2002; Harleman 2000; Luster et al. 1992; Schuurman

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et al. 1994) but the first official recommendations for enhanced histopathology were published in 2005 by the Society of Toxicologic Pathology (STP) Immunotoxicology Working Group (Haley et al. 2005) and the European STP (Ruehl-Fehlert et al. 2005). The need for standardization of immunohistopathology review was due, at that time, to the recently finalized guidance documents from European and US regulatory agencies that stated that immunotoxicity testing should be performed on all new investigational drugs or medicinal products (CPMP: Note for Guidance on Repeated Dose Toxicity; FDA: Guidance for Industry, Immunotoxicology Evaluation of Investigational New Drugs). The STP and ESTP thus provided guidance for a standardized and comprehensive "best practice" approach for the evaluation and reporting of lymphoid tissues using the enhanced histopathology approach. However there was much concern in the toxicologic pathology community in terms of how the evaluations should be done and the additional time and expense involved. In response, a series of articles were published that provided general guidelines on enhanced histopathology of lymphoid tissues using standard hematoxylin and eosin (H&E) stained sections (Elmore 2006a, 2006b, 2006c, 2006d, 2006e).

8.2 Enhanced Histopathology Defined

Enhanced histopathology is a tool that the pathologist can use to provide a more comprehensive evaluation of the immune system lymphoid organs (Haley et al. 2005). It involves the individual evaluation of the various compartments and subcompartments of each lymphoid organ with respect to size, cellularity, and other abnormal cellular changes such as apoptosis, necrosis and fibrosis (Table 8.1). It also involves the use of semiquantitative descriptive terms rather than interpretive terms to describe the cellular and architectural changes (Table 8.2). It should always be used in conjunction with gross changes, body weights, organ weights, clinical chemistry and hematological measurements.

The advantage of such a detailed and comprehensive evaluation is that it allows for an understanding of the changes that can occur with cell production and cell death as well as with cellular trafficking and recirculation following administration of immunomodulatory drugs and chemicals. Moreover, by identifying the specific compartments affected, one might be able to have an early indication of which cell population is affected and also provide clues on the possible mechanism of action of the test article. This methodology can be used with short-term studies (28 or 90 day) when routine histopathology indicates that there may be an immunomodulatory effect or there is a reason to suspect an immunomodulatory effect based on prior information such as previous studies or chemical structure. Enhanced histopathology may also be a part of a larger immunotoxicity protocol with a tier approach that includes traditional immune functional and nonfunctional tests. The final conclusions and interpretations should be a part of the pathology narrative.

Table 8.1 Lymphoid organ compartments and subcompartments

Thymus	Cortex and medulla
Spleen	Red pulp White pulp Periarteriolar lymphoid sheaths (PALS) Follicles/germinal centers Marginal zone
Lymph node	Cortex Follicles +/− germinal centers Interfollicular area Subcapsular sinuses Paracortex (deep cortical unit) Deep cortical unit Transverse sinuses Medulla Cords Medullary sinuses
MALT[a]	Follicles Interfollicular area
Bone marrow	One compartment

[a]MALT: mucosa associated lymphoid tissue

Table 8.2 Examples of interpretive and descriptive terminology

Interpretive terminology used in conventional evaluations	Descriptive terminology used in enhanced histopathology evaluation
Hyperplasia	Increased cells (indicate location and cell type)
Hypoplasia	Decreased cells (indicate location and cell type)
Atrophy	Decreased cells (indicate location and cell type)

Enhanced histopathology is not recommended for chronic toxicity/carcinogenicity studies because the inherent age associated changes that occur within some lymphoid organs may obscure or interfere with the determination of a treatment related immunomodulatory effect. As an example, the thymus, a common target of immunomodulatory chemicals, shows normal atrophy (age related involution; Fig. 8.1a, b) over time and medullary B cell proliferation can be a component of this normal change (Fig. 8.1c). If a potential immunomodulatory effect is suspected in a chronic study, then a repeat short-term study using enhanced histopathology is recommended.

8.3 Methodology and Organ-Specific Examples of Enhanced Histopathology

There are three primary points to consider for enhanced histopathology: Each lymphoid organ has separate compartments that support specific immune functions, these compartments (Table 8.1) should be evaluated individually and semi-quantitative

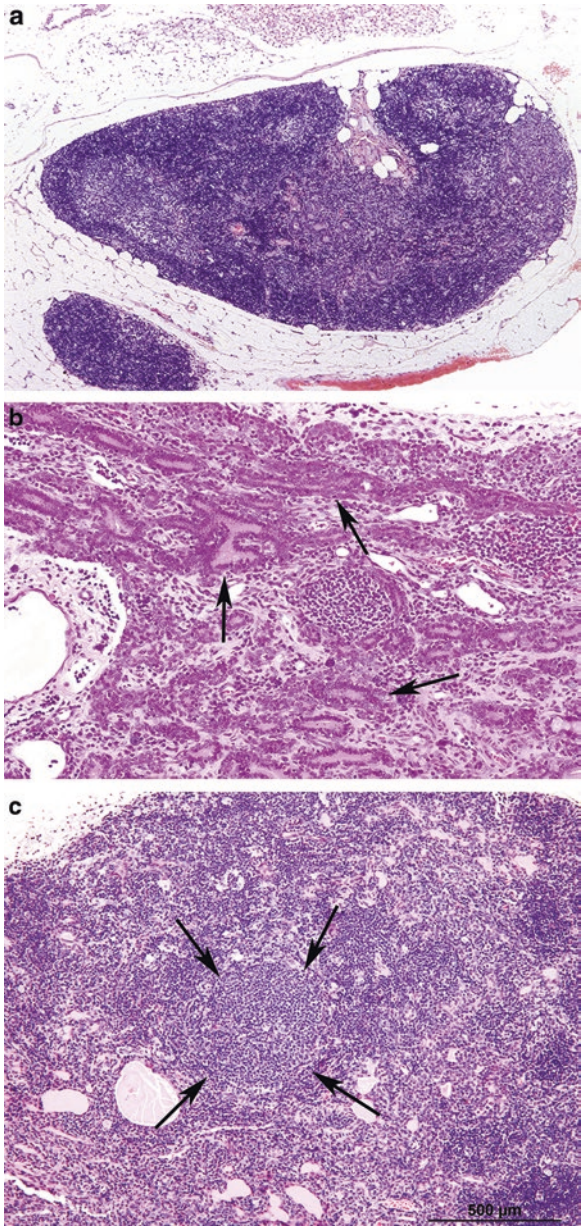

Fig. 8.1 (**a**) Thymus from a 2-year-old F344/N male rat with normal age related physiological involution. Note the small size, adipocyte infiltration, decreased cortex area and increased epithelial structures. H&E stain, objective magnification 2×. (**b**) Thymus from a 2-year-old F344/N male rat with epithelial tubule formation (*arrows*) associated with normal age related physiological involution. H&E stain, objective magnification 20×. (**c**) Thymus from a 2-year-old male mouse with focal B cell hyperplasia (*arrows*) associated with normal age related physiological involution. H&E stain, objective magnification 10×

descriptive rather than interpretive terminology should be used to describe the changes (Table 8.2) (Haley et al. 2005). The lymphoid tissues that should be evaluated include the thymus, spleen, select lymph nodes, bone marrow, and select mucosa associated lymphoid tissue (MALT). The choices of which lymph nodes and MALT to evaluate would depend on the route of administration and any other protocol specific

requirements. Detailed descriptions of drainage patterns for rat and mouse lymph nodes have been previously published (Sainte-Marie et al. 1982; Tilney 1971). The most proximal regional lymph nodes draining the site of application should always be examined. As an example, the nasopharynx associated lymphoid tissue (NALT) and bronchus associated lymphoid tissue (BALT) would always be examined for inhalation studies and the gut associated lymphoid tissue (GALT), or Peyer's patches, would always be examined for gavage or feed studies. Distal lymph nodes may be examined for evidence of a systemic effect.

During histological evaluation, the separate compartments are evaluated and semiquantitative descriptive terms are used. This is in contrast to the traditional interpretive terms such as hyperplasia, hypoplasia or atrophy. Also, the specific cell type is reported. For example, one would report "lymphocytes, increased" rather than "hyperplasia" and indicate the specific compartment(s) affected (Table 8.2). Also, increases or decreases in areas of compartments are reported. Additional abnormal findings such as necrosis, granulomas, inflammatory cells, mineralization, etc. are also reported.

8.3.1 Evaluation of the Thymus

The thymus is a primary lymphoid organ, essential for the normal development and function of the immune system (Elmore 2006a; Pearse 2006). The thymus is composed of two lobes, connected by a narrow isthmus and invested by a thin capsule. Each lobe contains numerous lobules that are divided by septae. Each lobule contains a darkly basophilic cortex and a paler staining medulla (Fig. 8.2a). The majority of cells in the thymus are T lymphocytes, which are subjected to a process of differentiation and maturation. Apoptosis is a common feature of these lymphocytes as they undergo positive and negative selection during the process of immunologic tolerance (Fig. 8.2b; Klein et al. 2014). The cortex and medulla should each be evaluated for increased or decreased compartment size and increased or decreased number of lymphocytes. Increases in apoptotic cells and/or tingible body macrophages would also be noted. Necrosis or other abnormalities would be noted if present. Immunohistochemical stains may be needed to better evaluate the lymphoid and non-lymphoid cells present in the thymus (Van Ewijk 1984).

Some rat strains have an additional compartment, the epithelial free area (EFA), which is postulated to be a site of lymphocyte storage, proliferation, or transit (Bruijntjes et al. 1993). These areas are lymphocyte-rich, lack stromal elements, and are non-vascularized. EFAs are located in the subcapsular region and can run deep into the cortex (Fig. 8.3a, b). The presence and amount of EFAs can vary with rat strain and age so comparison with concurrent controls is important. A stromal or endothelial stain, showing the lack of stroma and vascularity, may help to better identify these regions (Fig. 8.3c). For a more detailed and comprehensive discussion on the thymus, see Chap. 13.

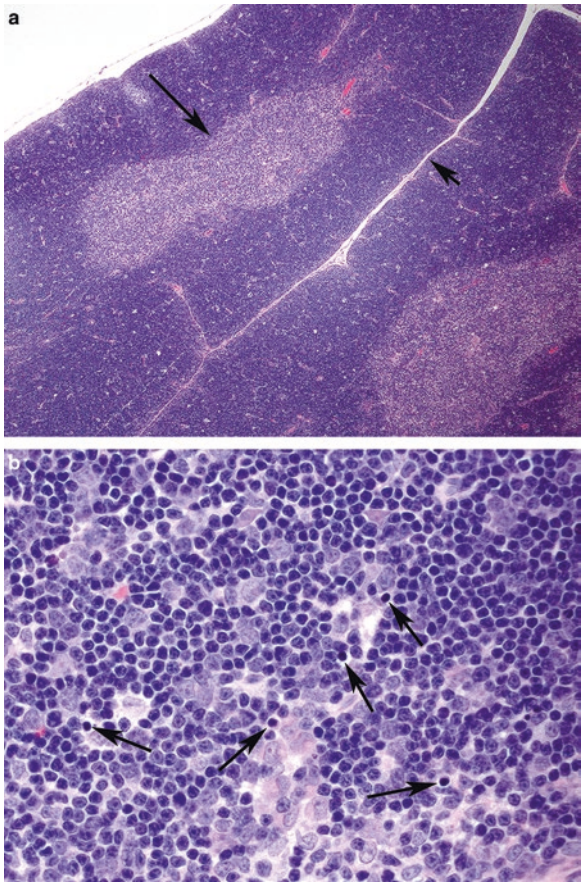

Fig. 8.2 (**a**) Thymus from a 3-month-old male Sprague Dawley rat. Note the lobules separated by an interlobular septum (*short arrow*) and the dark-staining cortex surrounding the paler eosinophilic medullary region (*long arrow*). H&E stain, objective magnification 4×. (**b**) Thymus from a 3-month-old male Sprague Dawley rat. Within the cortex and corticomedullary junction there are scattered apoptotic T lymphocytes characterized by small, round, dense hyperchromatic nuclei and scant or no cytoplasm (*arrows*). H&E stain, objective magnification 40×

8.3.2 Evaluation of the Bone Marrow

The bone marrow is the largest primary lymphoid organ, containing hematopoietic stem cells, which give rise to circulating leukocytes, lymphocytes, erythrocytes and thrombocytes. These cells can also repopulate lymphoid organs when needed. The bone marrow also functions in fat storage. Unlike the other lymphoid organs, the bone marrow has just one anatomic compartment (Elmore 2006e; Travlos 2006; Fig. 8.4). However, the same level of detailed evaluation should still be done in terms of identifying increases, decreases, or changes in maturation of the various cell types. These cell types include the erythroid, myeloid, megakaryocytic and stromal cells. Adipose tissue and hemosiderin-laden macrophages are also evaluated. However, the evaluation of lymphocytes cannot be done using conventional H&E stained sections. Therefore a more comprehensive evaluation of the bone marrow would include cytological preparations. An estimation of the myeloid:erythroid (M:E) ratio can be done with H&E but clinical pathology would be required to confirm and better define abnormal changes. Cellular changes in the bone marrow should always be compared with the cytology slide preparation and CBC to provide additional information regarding which cell type is increased or

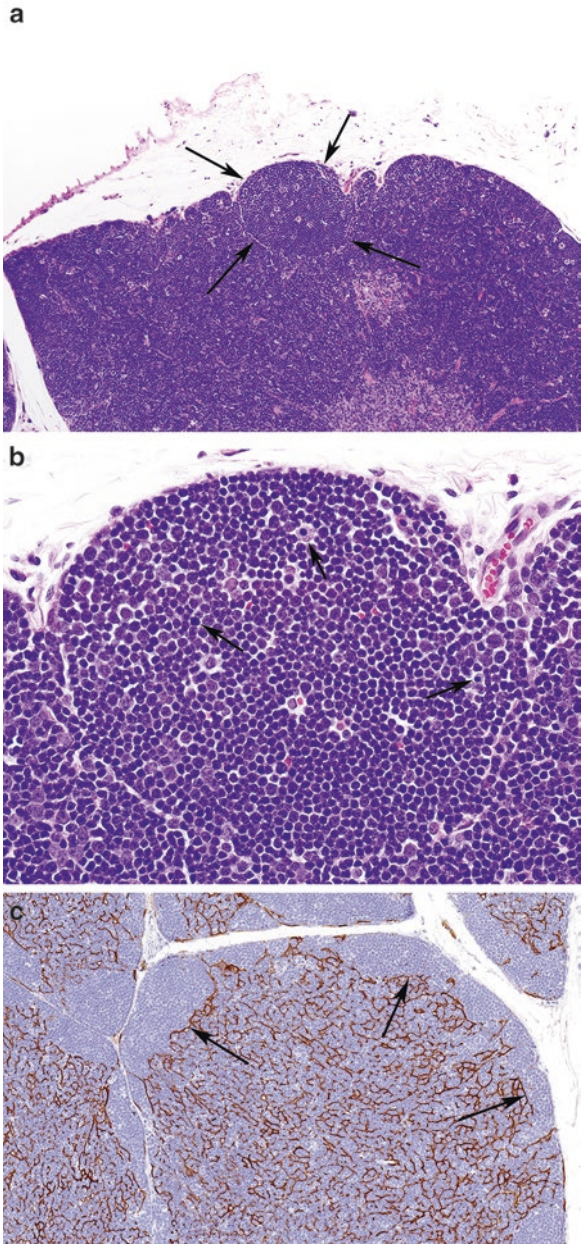

Fig. 8.3 (**a**) Low magnification of an epithelial free area (EFA) in a thymus from a 14-week-old Sprague Dawley rat. EFAs are sometimes present in the thymus cortex of various strains of rats. They are found at the subcapsular region and can run deep into the cortex. The function of an EFA is unknown. In this example, the EFA is outlined by arrows. H&E stain, objective magnification 10×. (**b**) High magnification of an epithelial free area (EFA) in a thymus from a 14-week-old Sprague Dawley rat. EFAs are characteristically composed of a higher than normal density of predominantly small CD4+CD8+ T lymphocytes. Apoptotic cells (*arrows*) and tingible body macrophages may also be present. Importantly, EFAs lack stromal elements so special stains, such as cytokeratin, may be used to evaluate these regions if needed. H&E stain, objective magnification 40×. (**c**) Epithelial free areas in the thymus of a 14-week-old Sprague Dawley rat are identified as non-staining areas with cytokeratin 18 (arrows)

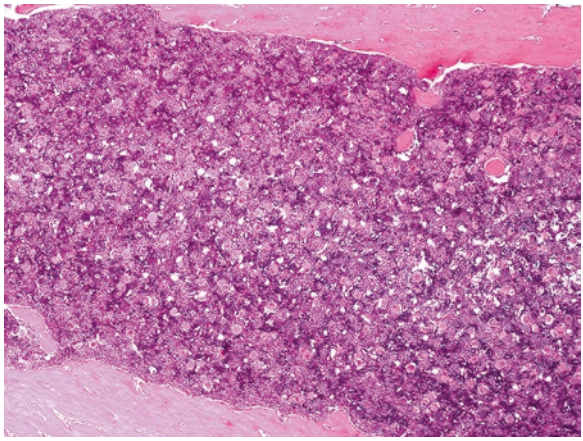

Fig. 8.4 Bone marrow from the femur of a 3-month-old male Sprague Dawley rat. At this age, the bone marrow at this location is typically cellular with little or no adipocytes. The marrow is composed of both myeloid and erythroid lineages. H&E stain, objective magnification 10×

decreased and to what degree. A maturation index is the ratio between the number of proliferative phase cells to the number of maturation phase cells and is not a component of enhanced histology. Enhanced histology may indicate a potential change in the maturation index, but differential counts of cells in marrow smears would be needed to better define the nature of the hematopoietic abnormality. For a more detailed and comprehensive discussion on the bone marrow/hematopoietic system, see Chap. 16.

8.3.3 Evaluation of the Spleen

The spleen is the largest secondary lymphoid organ and the primary function is as a blood filter. There are two major compartments of the spleen: the hematogenous red pulp and the white pulp (Cesta 2006a; Elmore 2006b). The subcompartments of the white pulp are the T cell rich periarteriolar lymphoid sheath (PALS), the B cell rich follicles and the macrophage rich marginal zone (Fig. 8.5). The red and white pulp should be evaluated for an increase or decrease in size and/or lymphocytes as well as an increase in numbers of cells such plasma cells, apoptotic cells, tingible body macrophages, dendritic cells, pigmented macrophages, etc. The red pulp would also be evaluated for an increase or decrease in hematopoietic cells as well as the diffuse lymphoid cell population. The follicles should be evaluated for an increase or decrease in germinal centers. Any other pathology in the spleen, such as fibrosis or necrosis, would be noted and the location indicated. For a more detailed and comprehensive discussion on the spleen, see Chap. 15.

8.3.4 Evaluation of the Lymph Nodes

The lymph nodes are the major route of entry for antigen and pathogens via the afferent lymph flow and are major activation sites of B, T and other immune cells. Acting as filters for foreign particles and cancer cells, lymph nodes are important for the

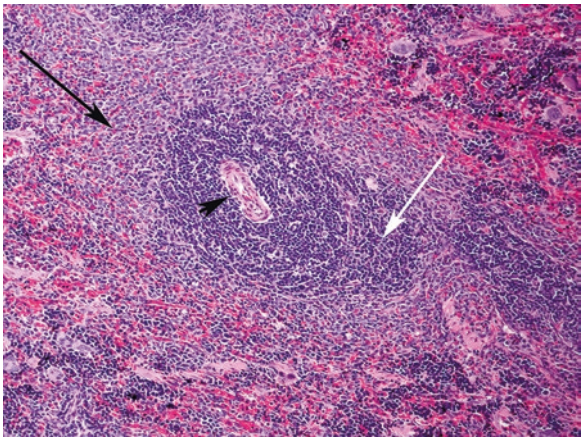

Fig. 8.5 Spleen from a 3-month-old male Sprague Dawley rat. This is a high magnification image of a PALS region with a central arteriole (*short black arrow*) surrounded by a densely cellular T cell rich periarteriolar lymphoid sheath (PALS) and that is surrounded by a paler macrophage rich marginal zone (*long black arrow*). A *white arrow* indicates the B cell rich follicle, present adjacent to the PALS region. H&E stain, objective magnification 20×

proper functioning of the immune system. There are three major functional areas in the lymph nodes: the cortex, paracortex and medulla (Elmore 2006c; Willard-Mack 2006; Fig. 8.6a) that support very specific immune functions. The cortex contains B cell rich follicles, with or without germinal centers (Fig. 8.6b), an interfollicular area of cellular trafficking, and subcapsular sinuses (Sainte-Marie and Peng, 1982). The paracortex contains the T cell rich deep cortical unit and the transverse sinuses (Belisle, Sainte-Marie and Peng 1982). The medullary region contains the B cell and plasma cell rich cords and the medullary sinuses. All of these compartments and subcompartments should be evaluated for any increase or decrease in cellularity or size. For a more detailed and comprehensive discussion on the lymph nodes, see Chap. 18.

8.3.5 Evaluation of the Mucosa-Associated Lymphoid Tissue

The mucosa-associated lymphoid tissues (MALT) are specialized mucosal epithelial surfaces of the body with aggregates of nonencapsulated organized lymphoid tissue and are responsible for local immune responses. Commonly evaluated MALTs include the bronchus associated lymphoid tissue (BALT), nasopharynx associated lymphoid tissue (NALT) and gut associated lymphoid tissue (GALT) (Breel et al. 1988; Elmore 2006d; Kuper et al. 1990; Kuper 2006). For many foreign pathogens, the MALT is the first line of defense. These lymphoid organs are composed of a loose aggregation of lymphoid cells and, depending on location and/or stimulation, may or may not have follicles and an interfollicular area (Cesta 2006b; Fig. 8.7a, b). There is follicle-associated epithelium (FAE) on the mucosal surface. Scattered within the FAE are microfold or M cells that cannot be evaluated with H&E stains, but that are

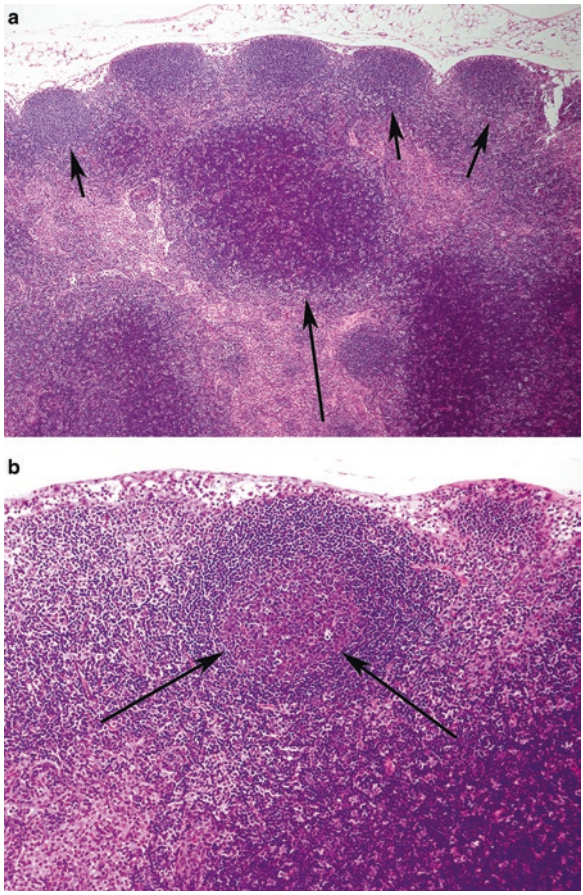

Fig. 8.6 (**a**) Mesenteric lymph node from a 3-month-old male Sprague Dawley rat. The paracortex (*long arrow*) and lymphoid follicles (*short arrows*) can be seen in this image. H&E stain, objective magnification 4×. (**b**) Lymphoid follicle within a mesenteric lymph node from a 3-month-old male Sprague Dawley rat. Germinal centers can develop in lymph nodes after exposure to antigen. The central activated germinal center (*arrows*) contains paler staining lymphoblasts as well as apoptotic cells. H&E stain, objective magnification 20×

important in the transport of antigenic material, such as organisms and particles, from the lumen to the underlying immune cells and are therefore important in stimulating mucosal immunity (Kraehenbuhl and Neutra 2000). The follicles and interfollicular area should be evaluated for an increase or decrease in compartment size or numbers of lymphocytes. The follicles should also be evaluated for changes in follicle and germinal center numbers. Other changes to note are hypertrophy of the high endothelial venules (HEVs), ulceration of the FAE, as well as location and severity of necrosis, plasma cells, granulocytes, pigmented macrophages, etc. For a more detailed and comprehensive discussion on the MALT, see Chap. 17.

8.4 Points to Consider and Caveats

Before evaluation of any lymphoid organ, one must have a thorough knowledge of normal structure, function and histology, including species differences (Elmore 2012; Haley 2003). There must be an understanding of what is normal before

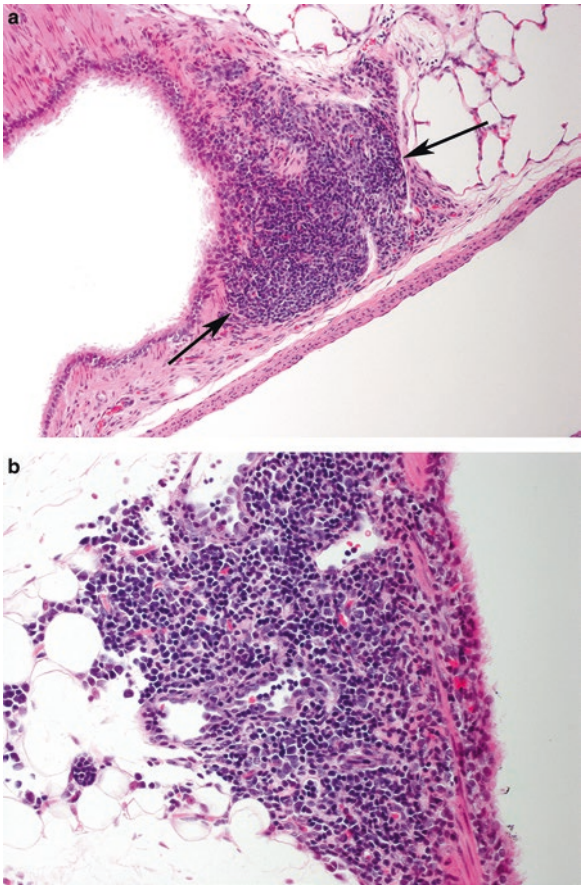

Fig. 8.7 (**a**) Bronchus associated lymphoid tissue (BALT; *arrows*) in the lung from a 3-month-old male Sprague Dawley rat. BALT is an organized aggregate of lymphocytes within the bronchial submucosa, randomly distributed along the bronchial tract. They are consistently present around the bifurcations of bronchi and bronchioles and always lie between an artery (lower right side of the BALT) and a bronchus (left side of the BALT). The majority of lymphocytes are T cells. H&E stain, objective magnification 20×. (**b**) Nasopharynx associated lymphoid tissue (NALT) from a 3-month-old male Sprague Dawley rat. In a rat, NALT is located in the ventral aspect of the lateral wall at the opening of the nasopharyngeal duct. In this image, the nasopharyngeal duct is to the right of the NALT. There is a mixture of B and T cells as well as plasma cells and macrophages. The NALT is the first line of defense for inhaled antigens. H&E stain, objective magnification 40×

abnormalities can be accurately diagnosed. For example, the marrow in aging animals is replaced with adipose tissue and the location of bone marrow sampling, such as the proximal versus distal femur, can have a dramatic effect on cellularity. The pattern of draining lymph nodes should also be known so that lymph nodes draining the site of application can be sampled as well as more distant lymph nodes, to look for systemic effects.

Because of the dynamic nature of the lymphoid system, there is the potential for a wide range of normal, especially in tissues exposed to dietary antigens such as the mandibular and mesenteric lymph nodes and Peyer's patches. There can also be a wide range of normal due to age, sex, species and strain. Therefore, a thorough review of all concurrent controls is necessary before evaluation of treated animals.

Diagnostic drift occurs when there are variations in the application of diagnostic terminology or criteria during the histopathological evaluation of a study. This is more likely to occur if the evaluation of the slides occurs over an extended period of time. Identification of the range of normal is an area particularly susceptible to diagnostic drift (Elmore 2012). One way to minimize diagnostic drift when enhanced histopathology is performed is to analyze one organ system at a time. As an example, one would first evaluate the thymus control tissues, then the high dose group, then the low and mid dose groups. Periodically re-evaluating a subset of control tissues as a "range of normal reference" can help to minimize diagnostic drift.

Blind scoring should not be done for the initial evaluation of tissues for enhanced histopathology. This is primarily because identification of the range of normal for each lymphoid organ is critically important. If subtle lesions are identified, then a secondary blinded review can be performed to confirm the findings.

Sectioning and staining are two other areas that should be given particular attention when performing enhanced histopathology (Elmore 2012). In general the largest surface area of a lymphoid organ should be prepared for evaluation. Facing the block to provide the largest surface area is necessary for some lymphoid organs, such as the spleen. The illusion of an increase in various compartments of an organ, such as the lymph node cortex, paracortex and medulla or the thymus cortex and medulla, might happen if superficial, tangential, or cross sections are prepared. Also, the entire chain of mesenteric lymph nodes should be collected and sectioned longitudinally because different lobules may drain different sections of the intestines. Sectioning tissues too thick or staining tissues too long may give an appearance of increased cellularity. There are other processing artifacts that could occur but one way to decrease these would be to section and stain all tissues together, preferably on the same day, by the same technician, and without changes in staining solutions.

Finally, it's important to use a holistic or "weight of evidence" approach for the evaluation of tissues using enhanced histopathology (Elmore 2012). The lymphoid system is dynamic, linked not only to other lymphoid organs, but also to other tissues in the body. Lymphoid organs are effector organs, becoming active in response to stimulation. Changes in a distant area or tissue could have a profound effect on the lymphoid tissue that is evaluated. For this reason, consideration of overall animal health status, environmental or endogenous stress, other organ involvement, hormonal changes, and other potential effects should be considered. Importantly, the methodology of enhanced histopathology does not directly measure immune function but, in conjunction with gross changes, body and organ weights, hematological measurements and clinical chemistry, does have the potential determine whether or not exposure to a xenobiotic may cause an immunomodulatory effect.

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Chapter 9
Clinical Pathology Assays in Immunopathology

Josely F. Figueiredo, Kirstin F. Barnhart, and Niraj Tripathi

Abstract Clinical pathology endpoints are evaluated during the course of toxicity studies to monitor structural and functional changes in organs and tissues in response to administration or withdrawal of a test item. A variety of assays can be performed in a clinical pathology laboratory with body fluids using either clinical pathology analyzers or other instruments. Information from the basic assays in clinical pathology, including hematology, clinical chemistry, urinalysis and coagulation are used with other safety assessment endpoints to evaluate immunotoxicity. Non-routine clinical pathology variables, such as acute phase proteins, cytokines, complement, hormones, autoantibodies, and bone marrow cytology are also included in preclinical toxicity studies as supplementary assays to ensure adequate interpretation of test item-related effects on the immune system. This chapter will describe the clinical pathology assays that are most useful to assess immunotoxicity.

Keywords Clinical pathology • Assays • Immunology

9.1 Introduction

Clinical pathology endpoints are an integral part of preclinical toxicity studies and are recommended by regulatory agencies to screen pathophysiologic alterations in response to administration or withdrawal of a test item (Tomlinson et al. 2013). The main regulatory guidances related to immunogenicity and immunotoxicity

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assessment include the Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs 2002, the ICH Harmonized Tripartite Guideline for Immunotoxicity Studies for Human Pharmaceuticals S8 2006, and the Committee for Proprietary Medicinal Products of the European Agency for the Evaluation of Medicinal Products 2000 (Kawabata and Evans 2012; Brennan et al. 2010) Interestingly, the ICH Guidance for Industry S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals contains minimal guidance on immunotoxicity. However, the ICH S8 guidelines, which were intended to provide guidance for low-molecular-weight drugs that are not intended to alter the immune system, actually contains many of the relevant principles and strategies for assessing immunotoxicity in chemical or biological test items.

In clinical pathology, the potential target organ toxicity is monitored by measuring changes in analytes present in body fluids that are related to tissue or cellular damage, altered metabolism and altered organ or cellular function. Clinical pathology testing generally involves collection of blood samples for hematology, clinical chemistry and coagulation assays and urine for urinalysis and occasionally for urine chemistry tests (Tomlinson et al. 2013; Weingand et al. 1996). These analyses contain a set of variables routinely analyzed in preclinical studies; some are relevant to immunotoxicity evaluation. Since the clinical pathology analytes are often not organ or cellular specific, alterations in these analytes and correlation with immunotoxicity must be interpreted by considering the laboratory animal species and its expected biological variation and physiological response, study design, temporal relationship between test item administration and lifespan of the analyte, correlation with related clinical pathology parameters and other endpoint findings. Therefore, clinical pathology assays provide valuable information to address immunotoxicity by an integrated interpretation of all data sets collected during a preclinical safety study.

The major clinical pathology variables altered by immunotoxicity are red blood cell count and related indices, leukocyte and platelet counts, serum total protein, albumin and globulin, and plasma fibrinogen. If immunotoxicity is suspected, other non-routine clinical pathology assays, such as acute phase proteins, complement, auto-antibody detection, cytokine analysis and bone marrow cytology can be included to ensure adequate interpretation of test item-related effects on the immune system. This chapter will discuss the routine and non-routine clinical pathology tests that can contribute to the immunotoxicity assessment.

9.2 Clinical Pathology Testing

9.2.1 Hematology

Hematologic changes are particularly important in identifying immunosuppression and immune stimulation (FDA 2002). For hematology testing, whole blood is collected in K2-EDTA tube (powder anticoagulant) or K3-EDTA (liquid anticoagulant). Although each is acceptable, K2-EDTA is preferable as it avoids potential

artifacts created by the liquid anticoagulant. The blood should be gently inverted and kept in a rocker until prior to analysis. Blood smears should be prepared within 6 h of phlebotomy (for most species), and samples should be analyzed using a validated hematology analyzer. Notably, rat red blood cells (RBCs) are very sensitive to the effects of storage in K2-EDTA, and smears ideally should be made within 30 min to avoid extensive echinocytosis and spherocytosis (Smith et al. 1994; Fry et al. 2014). Analysis by a hematology analyzer is performed optimally within 6 h of collection; however, minimal alterations in hematology variables have been reported in samples stored at 3 °C and analyzed within 24 h (Cora et al. 2012).

Hematology variable recommended by the Regulatory Affairs Committee (RAC) of the American Society for Veterinary Clinical Pathology (Reagan et al.), for routine toxicology studies in most laboratory species included hematocrit, hemoglobin, red blood cell (RBC) count, absolute reticulocyte count, mean cell volume (MCV), mean cell hemoglobin (MCH), MCHC (mean cell hemoglobin concentration), RDW (red cell distribution width), white blood cell count with absolute differential count and platelet count (coagulation parameter). When necessary, manual differential cell count can be performed on a case by case basis (Stockham and Scott 2008; Tomlinson et al. 2013). Automated hematology analyzers provide additional variables, such as mean platelet volume and reticulocyte hemoglobin content that can be utilized to investigate the mechanism of a test item-induced hematological changes. However, these additional variables are usually not included in standard hematology analysis and are not required by the regulatory agencies.

9.2.1.1 Leukocytes

In hematology variables, changes in leukocytes, specifically lymphocytes and neutrophils, are more relevant for immunotoxicity evaluation.

In general, decreases in circulating granulocytes and/or lymphocytes provide indirect evidence of myelosuppression. These indicators of myelosuppression including neutropenia or lymphopenia may reflect impairment of the immune system. However, a weight of evidence approach is recommended for proper evaluation of the leukocyte count and correlation with immune function where increased incidence of infection or tumors may provide corroborative evidence of immunosuppression (FDA 2002). Decreased leukocyte count, primarily lymphocytes, is also noted with stress or increased endogenous glucocorticoid concentrations. The effect of glucocorticoids on clinical pathology parameters is discussed in Sect. 9.2.6.

In the case of immune stimulation, the most common hematologic findings are increases in lymphocytes and/or granulocytes. The lymphocyte count is predominantly increased with biologic test items that are immunogenic; while the neutrophil, +/− monocyte and lymphocyte counts are increased with inflammation. However, significant immune system activation may occur without notable hematologic findings. Similarly, the total leukocyte count can double the value with excitement or frightening without any effect on the immune system. This process is called physiological leukocytosis and happens in response to epinephrine. Usually, the physiological leukocytosis

is transient and is seen during the acclimation period in those animals that are not used to handling. The cell type affected during the physiologic leukocytosis varies among species. In rodents, the lymphocyte count increases; in dogs, the neutrophil count increases while in non-human primates both neutrophil and lymphocyte counts increase with epinephrine response (Sahota 2013; Stockham and Scott 2008).

Neutrophils originate from myeloid progenitors in the bone marrow, and their release from the bone marrow depends on a balance between the chemokines CXCL1/2 and CDCL12. Neutrophils are directed to circulation by CXCL 1 and 2 which are highly expressed in the vasculature of the bone marrow and are retained in the bone marrow by CXCL12 which is produced by endosteal osteoblasts. Under inflammation, cytokines alter this balance and stimulate neutrophil mobilization from the bone marrow to circulation. Once in circulation, neutrophils can be part of the free-flowing pool or the marginated pool, which is mainly concentrated in spleen, liver and bone marrow (Saverymuttu et al. 1985; Suratt et al. 2001; Tak et al. 2013).

The lifespan of circulating neutrophils under physiological conditions has been discussed between researchers and varies from 11.4 h for mouse neutrophils (Basu et al. 2002) to 5.4 days for human neutrophils, depending on the method used for evaluation (Pillay et al. 2010). The plasticity of neutrophils plays a role in neutrophil migration during infection, inflammation and cancer immunology (Kolaczkowska and Kubes 2013). Neutrophil plasticity has been documented by the presence of different nuclear morphologies, and receptor and/or cytokine expression patterns when mice were infected with methicillin-resistant *Staphylococcus aureus* (Tsuda et al. 2004). It was also evident when different neutrophil subsets, specifically pro-inflammatory neutrophils (CD11b+Gr1+ CXCR4low) and pro-angiogenic neutrophils that expressed high levels of matrix metalloproteinase 9 and CXCR4, were recruited to tissues in response to pro-inflammatory chemokines or vascular endothelial growth factor A (VEGFA) (Christoffersson et al. 2012).

Inflammation activates neutrophils and increases their life span both in circulation and in tissues. In mice, the tissue lifespan can increase to 7 days with inflammation (Cheretakis et al. 2006). Studies with both zebrafish and mice demonstrated that tissue neutrophils can go through a process of reverse neutrophil migration and return to circulation by downregulation of junctional adhesion proteins (mice). This process could result in dissemination of inflammation to other organs leading to systemic and/or chronic inflammation (Kolaczkowska and Kubes 2013).

Neutrophils are recruited to tissues by macrophages, mast cells, platelets, epithelial cells and other tissue neutrophils by corresponding chemokines. In addition to their pivotal role in controlling infection, tissue neutrophils are known to modulate the adaptive immune response by mediating suppression of T cell proliferation and activity and to stimulate adaptive immunity by promoting humoral immune response through release of B cell-stimulating molecules (B cell–activating factor and CD40 ligand), activation of splenic B cells and by acting as antigen-presenting cells (Kolaczkowska and Kubes 2013).

Lymphocytes are one of the major participants in the immune system and their cellular biology and function will be described in other chapters in this book. Studies

of lymphocyte kinetics, indicate a circulating time (lifespan) of about 80 days for human T lymphocytes (Hellerstein et al. 1999) and about 18 days for B lymphocytes (Macallan et al. 2005). The standard hematology assays in clinical pathology provide the absolute lymphocyte count, which can be helpful for an initial interpretation and evaluation of the immune system; however, it must be integrated with other clinical and anatomic pathology findings for proper interpretation. Since lymphocytes continuously circulate between the lymphoid organs and blood, and are responsible for immune surveillance and constantly changing under stimuli, further evaluation of circulating and tissue lymphocytes by flow cytometry is considered a fundamental biomarker in immunopathology.

Monocytes are also essential components of the innate immune system and primary function by differentiating into macrophages and dendritic cells and by releasing cell signaling molecules. Under physiologic conditions, human monocyte lifespan in circulation is 1–3 days (Ziegler-Heitbrock 2000) where they can enter tissues to replenish tissue macrophage populations or undergo apoptosis (Yona et al. 2013). Their lifespan in circulation increases with inflammation, and the life span of tissue macrophages is much longer after differentiation (approximately eight months for alveolar macrophages) (Murphy et al. 2008). Monocytes are highly plastic and heterogeneous and change their functional phenotype in response to environmental stimulation. Two monocyte subsets with different functions were identified in mice according to expression level of an inflammatory monocyte marker Ly6C (lymphocyte antigen 6C), as $Ly6C^{+}$ (subdivided in high and meddle) and $LY6C^{-}$ or $Ly6C^{low}$ (Yang et al. 2014). $Ly6C^{+}$ monocytes are proinflammatory, have a high antimicrobial capacity and are involved in phagocytosis. In addition, $Ly6C^{+}$ monocytes are preferentially recruited into inflamed tissue and more likely to mature to inflammatory M1 macrophages. The $LY6C^{-}$ monocytes patrol the vasculature to monitor pathogen-associated molecular patterns (PAMPs) and secrete anti-inflammatory cytokines upon infection. They are recruited to tissue in vascular inflammation and differentiate into M2 macrophages contributing to tissue repair. (Leavy 2011; Yang et al. 2014; Yona et al. 2013). It has been reported that in steady state, $Ly6C^{+}$ monocytes control the lifespan of $Ly6C^{-}$ monocytes in the bone marrow, peripheral blood and spleen. In the pathogenesis of diseases, inhibition of inflammatory monocyte recruitment was shown to dampen detrimental inflammation and a potential strategy for treating inflammatory diseases (Leuschner et al. 2011). On the other hand, reduced phagocytic capacity of monocytes has been reported in patients with rheumatoid arthritis and cutaneous vasculitis (Hurst and Nuki 1981).

Monocytes are rapidly recruited to the tissue where they differentiate into tissue macrophages or dendritic cells (Yang et al. 2014). However, it is important to note that under some inflammatory conditions, monocyte influx may only contribute partially to the expansion of local macrophage population because proliferation of tissue-resident macrophages is another contributing process (Jenkins et al. 2011). In addition, it was demonstrated in mice that tissue-resident peritoneal, splenic, lung and hepatic macrophages are established during the gestational period and independent from circulating monocyte recruitment (Yona et al. 2013).

Basophils are normally present in very low numbers (<1%) in peripheral blood of most laboratory species, except rabbits which can have 5–12% of basophils in circulation. Studies in mice, indicate an estimated lifespan of 60 h (Ohnmacht and Voehringer 2009). Basophils are the primary source of IL-4 and is hypothesized that they trigger the development of antigen-specific T helper type 2 immune responses (Min et al. 2012). Basophils may be difficult to differentiate by manual count, if the granules are poorly stained (Lilliehook and Tvedten 2011).

Similar to basophils, eosinophils represent a minor leukocyte population in circulation of healthy animals and have a lifespan ranging from 8 to 18 h in the blood. After migration into the tissues, the life span of eosinophils ranges from 2 to 5 days. However, *in vitro* cytokines can increase the eosinophil survival to 14 days or longer (Park and Bochner 2010). Eosinophils participate in immune responses through production of cytokines, presenting antigens, binding to Toll-like receptors and eliciting T-helper immune responses. In addition, recruited eosinophils to tissues are a source of reactive oxygenated species (ROS) and lipid mediators of inflammation and source of cytokines associated with tissue repair and remodeling. In cutaneous T-cell lymphoma, the Th2 type cytokines result in eosinophilia, extracellular granule protein deposition and increased IL-5 levels in the skin and/or blood (Lee and Rosenberg 2013).

9.2.1.2 Red Blood Cells

Red blood cells (RBC) are the most abundant cell type in circulation. The lifespan of RBC varies among species from 45 days in rats to 120 days in non-human primates. In addition to their major function, oxygen and carbon dioxide transport, red blood cells directly participate in immune complex reactions with bacteria, complement and antibodies, reactive oxygen species production and hemoglobin antimicrobial activity, and indirectly by modulating T cell proliferation and survival (Morera and MacKenzie 2011; Fonseca et al. 2003; Porto et al. 2001). Genomic and proteomic studies have identified a total of 1989 non-redundant human erythrocyte proteins and mRNA that are potentially related to cellular defense, signal transduction and immune response (Kabanova et al. 2009). In relation to pathogen complex, glycophorin-A, an erythrocyte cell surface glycoprotein, plays a role in pathogen recognition. Glycophorin-A carries pathogen-associated erythrocytes to spleen where these infected erythrocytes are cleared (Baum et al. 2002).

Red blood cell count and related RBC indices are provided by the automated hematology analyzer. Manual review of blood smears for RBC morphology can provide valuable information for evaluation of immune-mediated processes, particularly, immune-mediated hemolytic anemia. The presence of spherocytes (sphere-shaped RBCs that lack central pallor) and ghost cells in conjunction with low RBC count and high reticulocyte count (regenerative response) are characteristic of extra and/or intra-vascular hemolysis. In immune-mediated hemolytic anemia, the altered morphology of spherocytes is secondary to binding of antibody or com-

plement to the RBC membrane (Stockham and Scott 2008). Spherocytes can also result from a defect in membrane structural proteins, as seen in hereditary spherocytosis (Eber and Lux 2004). In ghost RBCs, cell membrane is visible but the cytoplasm is either completely lost or remains in a small amount. These cells are formed due to leakage of hemoglobin through the membrane pores after attachment of complement fixing antibodies (IgG and IgM) (Stockham and Scott 2008).

Reticulocytes are immature, nonnucleated RBCs that contain RNA and therefore still produce hemoglobin. Reticulocytes are visualized by new methylene blue staining, which detects RNA and mitochondria. New methylene blue is a supravital stain that must be mixed with whole blood containing living cells prior to preparation of the blood smear. On a Wright-stained blood smear, these same cells will appear blue or basophilic and are called polychromatophilic erythrocytes (Stockham and Scott 2008). A few reticulocytes are present normally in circulation in most laboratory species, but their number increases with a regenerative response. Due to a relatively short lifespan of RBCs, rodents typically have a higher percentage of circulating reticulocytes. The lifespan of reticulocytes is about 18 h in rats and mice (Loeffler et al. 1989).

9.2.1.3 Platelets

Platelets are mainly involved in hemostasis; however, they also participate in the immune response either as a target, in case of immune-mediated thrombocytopenia (low platelet count) or as active contributors of immune mediators, such as cytokines, proinflammatory molecules, immunoglobulin class switch and germinal center formation. Platelets also express toll-like receptors that link innate immunity with thrombosis (Li et al. 2012). The lifespan of platelets in rodents is about 4–4.5 days (Kuter 1997). Blood smear evaluation is helpful for verification of thrombocytopenia and to rule out artefactual decrease in platelet count due to platelet clumping.

9.2.2 Cytological Evaluation of Bone Marrow

Bone marrow is a primary hematopoietic organ with immune regulatory function based on the production and maturation of B cells, migration and selective retainment of innate and adaptive immune cells and production of cytokines (Zhao et al. 2012). The bone marrow can also be a target in toxicity studies due to a test item-effect on bone marrow-derived immune system progenitors cells resulting in immunosuppression (Food and Drug Administration 2006).

Bone marrow cytology can be performed in toxicological studies to further address changes in the standard hematology and histopathology. The quality of the bone marrow smears is critical for cell lineage classification and morphology evaluation. In toxicological studies, bone marrow is typically collected from the

femur or sternum for small animal species (rodents) and from rib or sternum for large animals (dog, cat, primate, rabbit, swine). The collection is typically performed at necropsy, as quick as possible using the paint brush, push slide or squash/pull technique. For a review of bone marrow cytology recommendations and smear preparation, the readers are directed to best practices publication by Reagan et al. (Reagan et al. 2011). The bone marrow smears can be assessed qualitatively or quantitatively or semi quantitatively. The qualitative assessment include evaluation of maturation and morphology of the different cell lineages. The quantitative assessment, include the qualitative assessment, plus an enumeration of all myeloid and erythroid precursors. Lymphocytes and plasma cells are also enumerated. Alternatively, a semi quantitative assessment can be performed where enumeration of the major cell types, lymphocytes, erythroid and myeloid precursors are counted (Reagan et al. 2011). The myeloid to erythroid ratio (ME ratio) can be provided either qualitatively (estimated ME ratio) or quantitatively by calculating the ratio of the sum of myeloid precursors and erythroid precursors. Other findings can also be detected during cytological evaluation, such as increased numbers of macrophages and erythrophagia (macrophages containing phagocytosed RBCs) and presence of matrix, necrosis or abnormal cells.

Bone marrow cytology in toxicological studies is interpreted in conjunction with hematology and histopathology, taking into consideration the study design and the physiological kinetic of hematopoiesis.

The myeloid precursors in the bone marrow are divided into storage, maturation and proliferation pools. The storage pool contains mature neutrophils that are released into circulation immediately after a stimulation (i.e. high tissue demand). If the maturation pool is intact and the stimulus persist the maturation pool will develop into mature neutrophils, which takes 2–4 days. If production of neutrophils needs to start from stem cells, it will take 5 days before effects (increase in neutrophil count) are seen in peripheral blood (Stockham and Scott 2008). Similarly, the erythroid precursors in the bone marrow are divided in proliferation and maturation pools. In mice and rats, the average time for erythropoiesis (from colony forming unit to reticulocyte) under minimal stimulation is 6.2 days and under maximum stimulation is 4.7 days (Loeffler et al. 1989). Megakyopoiesis differs from myelo and erythropoiesis because the megakaryoblasts undergo endomitosis (DNA replication without cell division) with an enlargement of the cytoplasma that break up into platelets (Stockham and Scott 2008). Megakaryopoiesis (from the earliest recognizable megakaryocyte precursors to platelet production) is about 2–3 days in rodents and 5 days in primates (Kuter 1997). The time required for megakaryoposis from a primitive progenitor cell to platelets is not known. In general, if a test item is affecting all hematopoietic lineages, the first changes noted in hematology are low reticulocyte and neutrophil counts followed by low platelet count. The recovery is also at the same order, reticulocytes and neutrophils are the first cells to return in circulation, followed by platelets (Sahota 2013).

9.2.3 Clinical Chemistry

Serum or plasma can be used for analysis of clinical chemistry tests; however, for plasma use of lithium heparin is recommended as the anticoagulant because it has lower interference with testing of ions other than sodium heparin (Tomlinson et al. 2013). The total protein and globulin concentrations of serum are slightly lower than the plasma because fibrinogen and other clotting factors, that are proteins, are consumed during clot formation and removed from the serum. In routine serum chemistry, globulin concentration is not directly measured, rather calculated by subtracting measured albumin from measured total protein. This estimation is generally sufficient for establishing trends in serum globulin levels. More sensitive and specific methods for measuring immunoglobulins such as ELISA, electrophoresis, automated immunoturbidimetric assays (Tvarijonaviciute et al. 2013), and multiplex assays (Luminex) are used for accurate quantitation and characterization of immunoglobulin isotypes. Globulins are separated into three major fractions based on their electrophoretic mobility; α, β, and γ globulins. The α and β globulins are comprised of large arrays of proteins including acute phase proteins synthesized by the liver, whereas γ globulins primarily include immunoglobulins and are produced by lymphocytes and plasma cells in lymphoid tissues.

9.2.3.1 Globulins

Even though increases and decreases in globulin concentration may reflect immunostimulation and immunosuppression, respectively; the total globulin concentration is not very sensitive or reliable indicator of immunomodulation or immunotoxicity. Globulins are comprised of a large array of proteins including acute phase proteins, so minor increases or decreases in immunoglobulin or a specific type of immunoglobulin may not be evident in total globulin concentration. Serum immunoglobulins are recommended as part of standard testing because changes in immunoglobulins concentrations can be useful in certain situations to better understand target cell populations or mechanism of action (Food and Drug Administration 2006). Decreases in globulins can indicate immunosuppression; however, this finding is considered an insensitive indicator, and a weight of evidence approach is recommended when establishing significance.

Increases in immunoglobulins can be observed with immune stimulation. However, additional tests are usually helpful in determining or further characterizing the immune stimulation. These additional tests may include assessment of leukocytosis and measurement of cytokines, acute phase proteins, and complement fractions because they provide corroborative evidence, and increases in these parameters are usually evident more quickly than immunoglobulins.

With immunosuppression, the globulin concentration may be decreased depending on the mechanism involved, and corroborative evidence may be present in other

data, such as histologic evidence of lymphoid depletion in lymphoid tissues, decreased lymphocyte count in blood, and clinical evidence of immunosuppression (sepsis, pneumonia, opportunistic infections etc.). Impaired immunoglobulin production can result in decreased globulin concentration which is often reflected as increased albumin:globulin ratio. In case of decreased globulin concentration, serum protein fractions can be analyzed using appropriate assays to identify specific portion of globulins being affected (FDA 2002). The FDA guidance for immunotoxicology evaluation of investigational new drugs (FDA 2002) mentions increased incidence of infections and lymphoproliferative tumors as indicators of immunosuppression in toxicity studies. It recommends that the cause of infections be determined in such cases as the infections caused by weakly pathogenic organisms could be an important indicator of unintended immunosuppression. However, the relationship between immunosuppression and tumors is complicated and controversial. In most cases, the increased incidence of tumors in standard 2-year rodent carcinogenicity studies is attributed to genotoxicity, hormonal effects, or other relatively well understood mechanism. Although a definitive mechanism may not be apparent for tumors in some cases and the potential role for immunosuppression should be considered. The ICH S8 guidance emphasizes that stress-related immune changes (corticosteroid-mediated changes resulting from toxicity or exaggerated pharmacology) should be differentiated from direct immunosuppression caused by the test item, as both can present with similar findings. For example, decreased circulating lymphocytes, decrease in thymus weight, decreased cellularity of thymus and/or spleen and lymph nodes and associated histopathologic changes are commonly observed with the stress response. The guidance also emphasizes that the evidence of stress should be compelling, including clinical signs, in order to justify not conducting further immunotoxicity testing (Food and Drug Administration 2006).

In general, measurement of immunoglobulins (or globulins) as well as other clinical pathology biomarkers (such as changes in hematopoietic and lymphoid cells) may be more predictive for test items that cause immunosuppression by cell necrosis or apoptosis (off target toxicity). However, the distinction between exaggerated pharmacology and off target effects may be less obvious for test items that are intended to modulate immune system for therapeutic effect (Food and Drug Administration 2006). Utility of globulin measurement is also questionable for large molecule biotechnology-derived pharmaceuticals (biological) drugs that may elicit antigen-antibody mediated hypersensitivity reaction or drug-specific autoimmunity. The ICH S6 guidance recommends measurement of antibodies associated with administration of large molecule biologicals for repeat dose toxicity studies that can aid in interpretation of results. These anti-drug antibodies can be neutralizing or non-neutralizing. The production of neutralizing antibodies can result in loss of drug exposure which may be reflected in clinical pathology-related pharmacodynamics or efficacy end points and needs to be correlated with immunogenicity data (ICH 1997).

Evaluation of possible pathologic changes related to immune complex formation and deposition is also recommended by ICH S6 (ICH 1997). Although these

immunogenic responses, antigen-antibody mediated hypersensitivity reaction or drug-specific autoimmunity, in nonclinical species are not predictive of immunogenicity in humans. Due to the idiosyncratic nature of these responses, immunotoxicolgic testing strategies for large molecule biological products are generally developed on a case-by-case basis, and routine tiered approach or a standardized testing approach for immunotoxicity assessment are not recommended. An investigative study report published by Heyen et al. 2014 on immune complex disease in non-human primates elicited increases in multiple biomarkers with immune complex formation, including cytokines, complement fractions, and acute phase proteins (Heyen et al. 2014). The albumin concentration was decreased in all animals following the immune complex formation but a consistent increase in globulin was not observed, confirming that globulins are not reliable markers for immune complex formation. Complement activation has also been reported with intravenous administration of immunoglobulins at high doses (Mollnes et al. 1998).

These hypersensitivity and immunogenic reactions often lack a dose response because appropriate antigen to antibody ratio is critical for binding and too high concentration of the drug does not elicit the response. Similar phenomenon as the "prozone" or "Hook" effect described for the assay interference where spuriously low results can occur with very high concentration of analyte/biological products (Mire-Sluis et al. 2004). Immune complexes can deposit in filtering organs and have been implicated in pathogenesis of many autoimmune diseases such as systemic lupus erythematosus. One common sequela of immune complex deposition is glomerulonephropathy (Heyen et al. 2014) which may be evident in standard clinical pathology testing by the presence of azotemia (increased urea nitrogen and creatinine in blood) and increased protein, specifically albumin, in the urine. However, azotemia reflects altered glomerular filtration rate which may be evident in severe cases but it is not a very sensitive or reliable indicators of renal injury. Azotemia can also occur with prerenal causes, such as relative dehydration due to hypovolemia.

Increased globulin concentration can also be observed with administration of immunoglobulins (monoclonal antibodies), especially with the intravenous administration. The increase in globulin concentration in such cases is usually dose-dependent and correlates with drug exposure data and pharmacodymanic effects. This increase in globulin due to "presence of test item" should be differentiated from the actual immune response against the test item where production of antidrug antibodies can result in decreased drug exposure and altered pharmacokinetic profile due to immune mediated clearance mechanism.

Immunoglobulins can be further separated into various types and subtypes as needed (such as IgG, IgM, IgA, IgE), however, this differentiation is not recommended for general immunotoxicology testing. An industry white paper published by a group of experts (Mire-Sluis et al. 2004), including representatives from the FDA and several major pharmaceutical companies, on scientific recommendations for the development of anti-product antibody immunoassays intended for preclinical or clinical studies, suggested that screening assays for anti-drug antibody detection should at least include detection of IgM and IgG. They recognized while the

presence of other classes, such as IgE and IgA, may have clinical sequelae, their detection was highly dependent on the disease and patient population. Discussion with regulatory authorities was advised for determining utility of measuring subclasses of immunoglobulins on a case by case basis.

9.2.3.2 Acute Phase Proteins

The first reaction of body to immunological stress is the innate, nonspecific response, preceding specific immune reactions (Gruys et al. 2005b; Gruys et al. 2005a). The acute phase proteins (APP) is part of the early-defense or innate immune system triggered by different stimuli including trauma, infection, stress, neoplasia, inflammation and immunomodulation and includes complex systemic reaction with the goal of reestablishing homeostasis and promoting healing (Cray et al. 2009).

APP are primarily synthesized by liver in response to inflammation or acute phase response (APR) and most of the APPs migrate to alpha or beta region of globulins when serum/plasma protein fractions are separated by electrophoresis. Based on their response during APR and inflammation, APPs that increase in concentration are categorized as "positive" APPs and those that decrease in concentration are categorized as "negative" APPs (Gruys et al. 2005a; Gruys et al. 2005b). For general reference, term APP usually refers to positive APPs that are synthesized by the hepatocytes under the influence of proinflammatory cytokines, such as IL-6 and IL-1, with IL-6 being a major player (Ahmed et al. 2012). The increased hepatic synthesis of these positive APPs results in downregulation/decreased synthesis of negative APPs. Some examples of common negative APPs include albumin, transferrin, transthyretin (TTR, formerly called prealbumin), retinol binding protein (RBP), and cortisol binding globulin.

Increased production of positive APPs proteins is a sensitive indicator of inflammation or APR related to immune stimulation or other mechanisms. In general, cytokines are the early mediators of such events and their increases can precede inflammatory leukogram or changes in APPs which can increase within a few hours and usually peak at 24–48 h after dosing/stimulus (Honjo et al. 2010). Even though nonspecific indicators of inflammation, these APPS are used for monitoring various disease conditions (cardiovascular, vasculitis, trauma, malignancy) and used for health monitoring in large animals (Petersen et al. 2004; Kilicarslan et al. 2013). Positive APPs are further categorized as major, moderate or minor, depending on the degree of increase upon stimulation. Traditionally, major APPs are those that increase 10- to 100-fold, moderate APPs increase two to tenfold, and minor APPs only exhibit a slight increase (Cray et al. 2009). Major proteins generally increase markedly within the first 48 h and often have a rapid decline after the triggering stimulus is removed due their very short half-life (Cray et al. 2009). Whereas moderate or minor APPs exhibit relatively slow increase and decline, and may also be observed during the chronic inflammation. Species differences exist between species, and the magnitude of change also depends on the nature of stimulus (Kilicarslan

et al. 2013). Most commonly utilized major APPs for humans and most laboratory animal species include C-reactive protein (CRP), serum amyloid A, and haptoglobin, with the exception of rats where α1-acid glycoprotein and α2-macroglobulin are major APPs (Cray et al. 2009; Petersen et al. 2004). As a negative APP, decreases in albumin can correlate with both acute and chronic inflammation, particularly in non-human primates.

Unlike other APPs, fibrinogen, which is both a clotting factor and a positive APP, is normally present in high concentration to maintain normal clotting function. Due to its high basal concentration, increases in fibrinogen are not very pronounced (≤10-fold), making it a moderate or minor reactant for most species. However, it's a reliable indicator of inflammation in most species and small increases are usually meaningful. Haptoglobin (Hp) is also used as marker for hemolysis because Hp is utilized in binding free hemoglobin to facilitate iron recycling. During hemolytic episodes, Hp concentration is usually decreased because of its consumption with rapid turnover of hemoglobin (Gupta et al. 2011).

Along with cytokines and complement activation, APPs can serve as indicators for immunostimulation but because of their low basal concentration they are not good indicators for immunosuppression. Fibrinogen is the only APP that can exhibit remarkable decreases from basal concentration but that often reflects coagulopathy related to immune-complex formation or other immunogenic responses. Localized injection-site reactions for subcutaneous administration of biologics may also indicate mild immune stimulation which can be monitored by APPs as well as other markers of inflammation. However, these localized reactions may not elicit a systemic response.

Measurement of CRP is performed by several methods, including immunonephelometric;immunoturbidimetric;immunoluminometricassays.Immunoturbidimetric method is more commonly utilized and can also be adopted for automated biochemistry analyzers (Roberts et al. 2001). In addition, ELISA and a new method based on time-resolve fluorometry (TRFIA) have also been developed (Ceron et al. 2005). Immunoturbidimetric assay and sandwich ELISA assays are frequently used for measuremernt of SAA (Ceron et al. 2005). Haptoglobin can also be measured by ELISA whereas some new methods such as, Nephelometric immunoassay, have been developed. A lot of these assays are based on human Hp so species cross reactivity should be critically evaluated and assay validation done for satisfactory performance before use.

9.2.4 Urinalysis

The routine urinalysis test generally include urine appearance, volume, specific gravity, reagent strip chemistry tests (pH, glucose, ketones, protein, occult blood and bilirubin) and sediment analysis (Hall 1992). Changes in urinalysis may correlate with immunotoxicity when evaluated in conjunction with other clinical

pathology and histopathology findings. Alterations in the urine sediment such as high numbers of white blood cell and bacteria may be associated with immunosuppression with consequent opportunistic infections. Positive blood and/or bilirubin reactions in conjunction with changes in hematology (low RBC count, hemoglobin, hematocrit and high reticulocyte count) may be associated with immune-mediated hemolytic anemia. Positive protein reaction may reflect changes in glomerular vascular permeability and inflammation.

The reagent strip method detects amino groups of negatively charged proteins when bound to the dye in the strip pad. Because albumin is more negatively charged than globulin, the strip assay detects albumin better than globulin (Stockham and Scott 2008). Quantitative evaluation of urine total protein, albumin and globulins, performed by chemistry analyzers or ELISA-based assays, should be considered when data provide critical information on efficacy and risk assessment (Tomlinson et al. 2013).

With glomerulonephritis and changes in glomerular vascular permeability, there may be increased protein in tubular fluid with consequent damages to the tubular cells. The pathogenesis of this process can involve many factors including lysosomal burst due to overloading of the tubular cells catabolic capacity, activation of tubule interstitial dendritic cells or activation of alternative complement pathway, resulting in stimulation of the immune response and damage of tubular cells (Kurts et al. 2013). The damage to tubular epithelial cells is evidenced by alterations in the routine urinalysis, such as changes in urine pH, presence of crystals, casts and renal tubular epithelial cells. Glomerular alterations with resulting tubular damage are additionally assessed by quantifying renal biomarkers such as beta-2 microglobulin and cystatin C, Kim-1, clusterin, TFF-3, RPA-1 (Fuchs and Hewitt 2011).

9.2.5 Coagulation

There are major points of intersection between coagulation and the immune system that are integrated in the overall interpretation of a nonclinical study. Cellular necrosis, bacterial endotoxin and complement activate the coagulation pathway, which in turn alters the endothelial barrier function promoting migration of leukocytes into tissues (Esmon et al. 2011). In addition, inflammation induces expression of tissue factor, a primary initiator of the coagulation cascade, which also exacerbates the inflammatory responses by stimulating production of cytokines, chemokines and adhesion molecule via protease-activated receptors (PARs) (Witkowski et al. 2016).

The coagulation parameters recommended by ASVCP-RAC (American Society for Veterinary Clinical Pathology—Regulatory Affairs Committee) for routine toxicology studies for most laboratory species, include prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen and platelet count (discussed in hematology section). For mice, platelet count is the only variable recommended due to limited blood volume (Tomlinson et al. 2013). Proper sample collection and

sample handling are critical for accurate coagulation results. Whole blood should be collected in trisodium citrate anticoagulant at a 9:1 ratio (blood:anticoagulant) in siliconized glass tubes or plastic tubes (Tomlinson et al. 2013; Stockham and Scott 2008). The amount of blood to anticoagulant is critical because excess amount of citrate with less volume of blood than required will result in prolonged coagulation time and vice versa. Even with the correct volume of blood, drastic changes in RBC mass (hematocrit, red blood cell count, hemoglobin) can alter the ratio of plasma to anticoagulant. For example, in animals with low RBC mass and consequently more plasma per volume of blood, the sample may result in a shortened coagulation times due to proportionally lower amount of citrate while animals with high RBC mass and consequently less plasma per volume of blood, prolonged coagulation time due to proportionally higher amount of citrate. After sample collection, the plasma should be separated from the blood cells as soon as possible (within 1 h) and analyzed within 4 h; or depending on the sample stability for the species, samples can be frozen at −60 °C to −80 °C (Stockham and Scott 2008; Tomlinson et al. 2013).

Fibrinogen, APTT and PT assays evaluate the end point formation of fibrin. The APTT assay addresses the intrinsic or surface-induced pathway that includes coagulation factors XII, XI, IX and VIII and common pathway that include coagulation factors X, V, II (prothrombin) and I (fibrinogen). The PT assay addresses the extrinsic or tissue factor pathway that includes factor VII and common pathway (factor X, V, II and I). Fibrinogen concentration in the blood reflects the balance between production and consumption of this analyte. In terms of immunological responses, increase in fibrinogen indicates inflammation as discussed in the acute phase protein section and prolonged APTT and PT may reflect disseminated intravascular coagulopathies secondary to sepsis or immune complexes (Nakamura et al. 1990).

Another important assay related to hemostasis is D-dimer, which is not part of the standard clinical pathology assay but has been investigated as a marker of prothrombotic conditions and inflammation in pre-clinical studies. This assay is based on detection of antigen Fibrin-D-dimer in plasma or whole blood to addresses fibrinolysis, the enzymatic degradation of fibrin to restore normal tissue architecture. D-dimers are measured by immunoassays by ELISA, immunogaglutination, immunochromatographic or immunophotometric and others platforms (Sathe and Patwa 2014; Adam et al. 2009).

Local fibrin formation and lysis are part of local inflammatory response and D-dimers has been shown to modulate acute phase responses and mediate systemic inflammation (Sathe and Patwa 2014; Adam et al. 2009). Although D-dimers are related to fibrin deposition and should be indicative of thrombus formation, in human, other conditions unrelated to thrombosis were identified to increase circulating concentrations of D-dimers, including neoplasms, infection, hemorrhage, congestive cardiac failure. Therefore due to the poor specificity of this assay, decision thresholds are established to correlate D-dimer concentrations with different conditions (Tripodi 2011). Similar approach may have to be established in laboratory animals due to the poor specificity of the assay and the species- and individual-related biological variability.

9.2.6 Hormones

Hormone evaluation is not part of the standard clinical pathology tests performed in non-clinical toxicolsogy studies; however, when available, it may help in understanding the mechanisms related to modulation of immune system.

The effects of hormones on immune function will be discussed in detail in the endocrine chapter. Briefly, it is currently known that a reciprocal relationship exists between the endocrine and immune systems in pathophysiological conditions or to maintain the physiological homeostasis. Hormone receptors are expressed on immune cell subsets and likewise, immune-mediator receptors are expressed on cells of the endocrine system. In addition, metabolic hormones, such as leptin and ghrelin, have been shown to be produced by immune cells, in addition to their primary production sites, adipocytes and stomach, respectively (Taub 2008). This relationship is also evidenced by the fact that thyroid stimulating hormone (TSH) was found to be produced by small intestine epithelial cells and to be a key immunoregulatory mediator in the intestine by regulating TSH receptor-expressing T cells in the mouse (Wang et al. 1997).

It is also known that pituitary, metabolic, sex and stress hormones are involved in regulating the immune function. Sex hormones can have immunostimulatory (e.g. testosterone) or immunosuppressive (e.g. estrogen) properties; pituitary hormones (e.g. growth hormone) can mediate proliferation of myeloblasts and T lymphocytes; metabolic hormones, such as ghrelin can act as an anti-inflammatory mediator while leptin can act as a pro-inflammatory cytokine. Leptin is also involved in the pathogenesis of autoimmune diseases, including diabetes and arthritis and is known to control metabolic activity by regulating T3 and T4 production (Taub 2008).

Stress hormones, particularly glucocorticoids (cortisol in human and large animal species and corticosterone in rodents) and catecholamines, also regulate the immune system. Physiological levels of glucocorticoids are considered immunomodulatory; whereas, high levels encountered during physiologic stress are immunosuppressive. It was demonstrated in mice that stress hormones reduce natural killer cell activity (Bonneau et al. 1991), alter antibody production, and modulate cytokine production (Webster Marketon and Glaser 2008). Addtionally, they can lead to ineffective DNA repair, which increases the likelihood of cancer formation.

In terms of the standard clinical pathology variables related to the immune system, glucocorticoids decrease the circulating lymphocyte count by affecting the survival and differentiation of T and B lymphocytes (Gruver-Yates et al. 2014; Cupps et al. 1985) and decrease the basophil (Yamagata et al. 2012; Yoshimura et al. 2001) and eosinophil counts by inducing apoptosis (Everds et al. 2013). Glucocorticoids also increase neutrophil counts by inducing demargination from blood vessels, increasing release from the bone marrow, and inhibiting apoptosis (Cox 1995; Saffar et al. 2011; Stockham and Scott 2008). Glucocorticoids stimulate antibody production in vitro and in vivo at physiological concentrations by shifting the immune response towards Th2 cytokine profile (Elenkov 2004); whereas, high

concentrations of glucocorticoids are lethal to non-differentiated B cells (Roess et al. 1982). Measuring stress-related blood hormones and their comparison with drug exposure may help to understand the role of stress in drug induced immunosuppression (Pruett et al. 1999; Pruett et al. 2000).

Hormones are most often analyzed in serum samples, but they can be analyzed in other matrices such as urine, cerebrospinal fluid, saliva and feces. Radioimmunoassay is a highly sensitive and specific method. However, the disadvantage of using radioactivity has resulted in a much higher demand for enzyme-linked immunosorbance, immunofluorescence and chemiluminescence based hormone assays. These assays are most often available as singleplex for laboratory species; however, multiplex has assays are available for some hormones. Liquid chromatography-mass spectrometry is also used, especially for small molecule hormones (Stanislaus et al. 2012). Hormone assays are often challenging to develop and validate for laboratory species because of their short half-lives, limited availability of species-specific assays, and the structural differences in hormones between species which prevents universal application of immunoassays across different species (Evans 2009). When requested in non-clinical toxicologic studies, certain peculiarities must be considered in the study designs in relation to sample collection time and number of animals needed for adequate statistical power (Stanislaus et al. 2012; Chapin and Creasy 2012).

9.2.7 Autoantibodies

Autoantibodies are the serological hallmark of autoimmune disease. They can be divided into two categories, those against organ or cell-specific antigens such as anti-thyroid, pancreatic beta-cell, erythrocyte or platelet autoantibodies and those against non-organ specific antigens such as anti-DNA-, anti-histone and anti-cytoskeletal antibodies that are involved in systemic autoimmune diseases (Verdier et al. 1997). In this chapter erythrocyte and platelet autoantibodies and some more common non-organ specific autoantibodies will be discussed. The pathogenesis of autoantibodies involve complement-mediated cell lysis and phagocytosis as seen in autoimmune reactions against platelets and RBCs and/or interaction with receptors or proteins or peptides as seen in systemic auto-immune diseases (Verdier et al. 1997). In the case of lupus, there is the formation of immune complexes, most often nuclear antigens/antibody complexes that are present in circulation or deposited in tissues, such as the kidneys, driving cytokine production (Verdier et al. 1997; Pisetsky 2016). The exposure of nucleoprotein complexes to the immune system is suspected to happen with cell death, either apoptosis, necrosis, necroptosis or NETosis (Pisetsky 2016) and subsequent activation of interleukins, complement cascade, Fcg receptors on inflammatory cells or intracellular Toll-like receptors (Pisetsky 2016; Colonna et al. 2014).

9.2.7.1 Anti-platelet Antibodies

Drug-induced immune thrombocytopenia (DITP) has been implicated in over 150 drugs administered to humans; however, reports of DITP in preclinical safety are extremely rare. Although DITP can be directly confirmed through laboratory testing to detect drug-dependent platelet-associated immunoglobulin (PAIgG), validated assays are difficult to achieve and not routinely available even in human medicine (Heikal and Smock 2013). Consequently, diagnosis of DITP is frequently achieved by considering not only established clinical criteria but also the temporal relationship to drug administration and the absence of associated laboratory abnormalities that would indicate other pathologic mechanisms (Heikal and Smock 2013; Kenney and Stack 2009). For cases in which clinical criteria alone cannot establish a diagnosis of ITP, laboratory testing remains an important tool, albeit one that is underdeveloped and performed only in a few specialized testing centers. In 2013, Arnold et al (Arnold et al. 2013a) proposed four specific criteria for the laboratory diagnosis of DITP: (1) antibody binding to platelets must be dependent upon the presence of the drug or metabolite, (2) immunoglobulin binding must be demonstrated through a quantitative method, (3) platelet specificity must be demonstrated, and (4) the results must be replicated by a second laboratory. It is important to consider that a drug metabolite may be the sensitizing agent when testing for anti-platelet antibodies. This scenario is more common than once thought and an important cause of false-negative results with the parent compound (Aster and Bougie 2007).

The diagnosis of DITP faces similar but even greater challenges in veterinary species as commercial laboratories that routinely perform anti-platelet antibody testing are extremely rare. If DITP is suspected in an investigative or toxicology study, it is often incumbent on the investigators to develop their own methods for detecting and quantifying PAIgG. In dogs, a direct immunoradiometric assay for measuring PAIgG has been previously described (Scott et al. 2002), and direct PAIgG measurement is currently provided by the Clinical Immunology section of the Kansas State Veterinary Diagnostic Laboratory (http://www.ksvdl.org/laboratories/clinical-immunology/).

Both direct and indirect methods of detecting PAIgG have been used; however, indirect methods that quantitate PAIgG in plasma or serum are not typically considered reliable due to a high degree of nonspecific binding, low sensitivity and the inability to differentiate between anti-platelet antibodies, anti-platelet alloantibodies, immune complexes and aggregates of normal IgG (Scott et al. 2002). Direct assays performed on washed and isolated platelets are preferred because they provide greater sensitivity and reproducibility, but they are more technically challenging, particularly when platelet counts are greatly reduced (Scott et al. 2002; Heikal and Smock 2013).

Direct methods that have been used to accurately detect and quantify PAIgG include flow cytometry, antigen-capture ELISA, enzyme immunoassay, the platelet antiglobulin test and others (Arnold et al. 2013b). Although direct assays for PAIgG are effective for diagnosing the general process of immune-mediated thrombocytopenia, more specific reference testing should be performed to conclude that the

anti-platelet antibodies formed secondary to test item administration. Typically, this is accomplished by incubating patient serum with isolated and wash platelets (from a normal donor) in the presence and absence of the test item followed by quantitation of bound immunoglobulin in each sample (Kenney and Stack 2009; Arnold et al. 2013a). Flow cytometry and enzyme immunoassay are the most commonly used and sensitive methods for detection of drug-dependent platelet antibodies (Kenney and Stack 2009).

Direct flow cytometric detection of platelet-associated immunoglobulin (PAIgG) was originally described in rhesus monkeys following nasogastric administration of 7,8-dimethoxy-1H-3-benzaepin-2-amine HCL in a chronic toxicology study. PAIgG was detectable by flow cytometry on isolated platelets from treated rhesus monkeys up to 3 weeks before onset of thrombocytopenia (Petersen et al. 1988). Bednar et al. (1999) used a gel-filtration platelet isolation technique followed by flow cytometric detection of drug-dependent antibodies to identify preexisting drug-dependent antibodies in a chimpanzee and rhesus monkey (Bednar et al. 1999).

9.2.7.2 Anti-erythrocyte Antibodies

Drug induced immune-mediated hemolytic anemia (DIIHA) is a rare side-effect of drug administration. Estimates in humans suggest an incidence of one to four cases per million individuals per year for small molecules and vaccines, although the incidence is likely higher given the number of undiagnosed cases. (Garratty 2010; Petz 2004; Klein et al. 2010; Mayer et al. 2015). In preclinical safety studies, the dog has been described as the most susceptible species to DIIHA (Haschek et al. 2010; McGrath 1993); however, the incidence in other large animal species may be underestimated due to the relatively high numbers of dogs used in small molecule toxicology studies. To date, greater than 130 marketed drugs have been associated with drug induced immune-mediated hemolytic (DIIHA) in humans (Garratty and Arndt 2014).

DIIHA can be caused by two types of antibodies: (1) drug-independent antibodies that can be detected in vitro without the presence of the drug and react like true autoantibodies; and (2) drug-dependent antibodies, which are only present in vitro in the presence of the drug (Garratty and Arndt 2007; Garratty 2009). Based on reports in humans, DIIHA is most commonly associated with drug-dependent antibodies (Pierce and Nester 2011). In 1947, Landsteiner demonstrated that drugs could become immunogenic when complexed with a carrier molecule, frequently a protein, and termed these drugs haptens. Subsequent work determined that anti-drug antibodies may be directed against the drug (hapten) alone, the RBC membrane, or a combination of the drug and the RBC membrane (Garratty and Arndt 2007). Anti-drug antibodies may also react to an epitope found on its metabolites (Pierce and Nester 2011).

Drug-dependent antibodies can be classified into two main subtypes based upon their in vitro activity: (1) antibodies that react to a drug that will remain on the RBC membrane after several washes, typically due to covalent binding, and; (2) drugs

that are associated with the RBC membrane but not covalently bound and cannot be detected by antibodies against drug-coated RBCs. In the second case, the antibodies are detected by combing the patient's serum that contains the unbound anti-drug antibody and compatible RBCs. Reports have also shown that a drug may induce antibody formation through a combination of these mechanisms (Garratty 2012).

Drug-independent antibodies have been documented in a minority of DIIHA reports in human medicine. The mechanisms for why these antibodies are formed are not well understood, but considerations include molecular mimicry, immune dysregulation and altered RBC membrane proteins through drug adsorption (Pierce and Nester 2011). In this latter mechanism, proteins such as immunoglobulin, complement and albumin attach to the RBCs and cause a positive antiglobulin tests (Garratty 2012). It is often difficult to conclusively determine that the immune-mediated hemolytic anemia is attributable to pre-existing autoantibodies, and a diagnosis is presumptively made by the presence of hemolytic anemia or a positive direct antibody test (DAT) during drug administration that resolves following cessation of drug therapy (Garratty and Arndt 2007). If serum is available prior to drug administration (which may be the case in a toxicology study), it may be tested for pre-existing drug-independent antibodies.

The original direct antiglobulin test (DAT) was described in 1908 (Moreschi 1908) but more fully described by a veterinarian, Robin RA Coombs, in 1945, and the method for detecting antibody and complement on the surface of the RBC was subsequently named after him. The Coomb's test can be performed as a direct antibody test (DAT) that detects immunoglobulin and/or complement on the surface of RBC or as an indirect antibody test (IAT) to detect unattached RBC antibodies in serum (Wardrop 2005; Zantek et al. 2012).

A positive result with a DAT is considered the most reliable finding for documenting DIIHA in humans (Pierce and Nester 2011). The DAT is optimally performed on EDTA anti-coagulated blood, and the DAT test tube has been the gold standard agglutination technique that typically uses polyspecific Coombs' reagents. Alternative platforms include the more sensitive microcolumn and solid phase methods have been developed. (Zantek et al. 2012). Positive reactions may be confirmed with monospecific reagents for immunoglobin (typically the gamma heavy chain portion) or complement. The most common anti-complement reagent, anti-C3d, is indicative of bound IgM (Zantek et al. 2012; Freedman 1987).

In preclinical species, commercially available DAT reagents and materials are not widely available. For many years, this method has been performed in dogs with varying degrees of success due to poor sensitivity, the lack of standardized monospecific reagents, and a high degree of variability in reaction conditions and methodology (Wardrop 2005). More recently, the development of assays in human medicine with enhanced specificity (e.g. microtiter plates, capillary tubes, gel columns and flow cytometry) and improved monoclonal reagent strategies has provided alternative strategies for developing more reliable DATs in the dog. In a recent report by Caviezel (2014), four separate methods for DAT in dogs were evaluated and two methods, the novel strip and capillary DAT, were identified as promising tools for diagnosis of DIIHA (Caviezel et al. 2014).

The IAT is used commonly in human medicine to detect incomplete antibodies against antigens present on donor RBCs prior to transfusion (Wardrop 2005). It may also be used to identify pre-existing drug-independent antibodies to a specific drug. In this case, the patient's serum is combined with an aliquot of the drug and incubated. A positive reaction is determined by the presence of agglutination. In veterinary medicine, the IAT is typically used to screen canine serum for RBC antibodies for blood banking and transfusion purposes (Wardrop 2005). It may also be used to detect drug-independent antibodies; however, one of the drawbacks to this assay in animals is the lack of well-understood blood classification systems in many species (Wardrop 2005). If an animal's serum has any pre-existing antibodies to donor RBCs prior to addition of the drug, it may cause a false positive reaction. If subsequent addition of serum from a second animal without exposure to the drug to the same donor RBCs does not result in agglutination, then more confidence can be placed on the interpretation of pre-existing anti-drug antibodies.

In humans, an elution is typically performed following a positive DAT to further characterize the antibody coating the RBCs. In this test, unbound antibody is washed from RBCs. Attached antibodies are then removed by chemical modification (usually acid). This eluate is then tested against reagent RBCs. If a negative reaction occurs, it is because the eluate does not contain the drug. A positive reaction is indicative of true autoimmunity (Pierce and Nester 2011).

Although many improvements have been made in all of the testing methods for DIIHA in both humans and pre-clinical species, a number of factors remain that can cause false-negative and false-positive results. Some of the common problems that contribute to false-negative results include incomplete washing, excessive agitation when reading the test tube, ineffective polyspecific reagents, and failure to use the optimal temperature. False-positive results may be generated by use of serum separator tubes, use of clotted blood samples, the presence of naturally occurring cold autoantibodies, and the presence of polyagglutinable RBCs from septicemic patients (Wardrop 2005).

9.2.7.3 Systemic Autoantibodies

The common autoantibodies used in human clinical practice used to detect systemic autoimmune diseases include rheumatoid factor, anti-cyclic citrullinated peptide (CCP) antibodies, antinuclear antibodies (ANAs), anti-neutrophil cytoplasmic antibodies (ANCA) and anti-phospholipid antibodies. These assays are infrequently used in routine preclinical safety assessment because the relevance or extrapolation of findings in laboratory animals to human is difficult (Verdier et al. 1997; Gad et al. 2008) and even studies with drugs known to cause autoimmune diseases in human did not result in autoimmunity in animals (Herzyk and Bussiere 2008). However, these assays are useful for evaluating new drug candidates with a potential to cause autoimmune diseases in autoimmune-prone animals and for investigating mechanism of autoimmune reactions during toxicology studies (Verdier et al. 1997).

Detection of ANA is a screening test for the presence of antibodies to all nuclear antigens, including DNA and RNA-associated proteins, centromere, nuclear membrane and nucleoli. Specific assays, such as the double-stranded DNA assay should be tested after a positive ANA test. (Aggarwal 2014). ANA and dsDNA tests are frequently performed by indirect immunofluorescence (IFI), which is considered the gold-standard method. In IFI the tested serum is put in contact with the antigen and the antigen-antibody complex is detected by a secondary fluorochrome-conjugated antibody. The antigens or substrates can be tissues, isolated cells or *Crithidia luciliae*, which is an organism that contains high concentration of dsDNA in its kinetoplast and is used as a substrate in the dsDNA test. The distribution of antigen-antibody complexes result in distinct fluorescence patterns that are associated with certain autoimmune diseases (Verdier et al. 1997; Kumar et al. 2009). ELISA-based assay is also used for detection of autoantibody. The assay can be generic to detect a broad range of antigens or specific assays for a single autoantigen. The radiolabeled assays are available and considered specific but technically difficult because of the use of radioactive material. Flow cytometry with autoantigen-coated fluorescent beads has been considered cost effective and a sensitive method to detect autoantibodies (Kumar et al. 2009). Rheumatoid factor (Leuschner et al.) is an autoantibody, usually IgM directed against aggregated immunoglobulin G (IgG). RF can be measured using latex agglutination, ELISA and nephelometry (Aggarwal 2014) through chemistry analyzers.

9.2.8 Complement

Immunogenicity, particularly to biopharmaceuticals may result in activation of the complement system. Given the significant increase in the number of biologics being developed in many therapeutic areas, the need to assess complement activity in preclinical studies is becoming more important. Currently, methods for evaluating complement are best established for the cynomolgus monkey.

Activation of the complement system occurs through three main pathways: classical, lectin and alternative, each leading to a common terminal pathway(Merle et al. 2015). During normal physiologic conditions, a low level of alternate pathway activity is constitutively present in serum while the classical and lectin pathways are primarily activated by exogenous stimuli (Merle et al. 2015) (Noris and Remuzzi 2013) including pharmaceutical agents. The most common types of compounds that cause complement activation include immunoglobulins, liposomes and lipid excipient-based therapeutics, and DNA/RNA based therapeutics, particularly antisense oligonucleotides (Shen et al. 2014; Tawara et al. 2008; Szebeni 2014).

The central component of the complement system is C3, the most abundant complement protein in serum (Mastellos et al. 2004). Activation of all three pathways occurs through the cleavage of inactive C3 into active C3a and C3b fragments, which function as potent pro-inflammatory mediator and an opsonin, respectively (Merle et al. 2015). These fragments are commonly measured in plasma as general

indicators of complement activation. C4 activation products are formed through the activation of the classical and the lectin pathways and are also commonly measured in plasma. Activation of the alternative pathway is dependent on the cleavage of Factor B into Ba and Bb activate fragments (Harboe et al. 2011). The terminal pathway is initiated by C5, which is most commonly activated secondary to C3 activation but direct activation by other mediators (e.g. kallikrein and thrombin) has also been described (Noris and Remuzzi 2013). C5a and C5b-9 are generated following C5 activation, and both products may be measured as an indication of terminal pathway; however, C5a has an extremely short in vivo half-life of approximately 1 min, while C5b-9 has a half-life of 50–60 min and is relatively stable in vitro (Bergseth et al. 2013; Kirschfink and Mollnes 2003).

Complement activation is evaluated through assays that assess the functional ability of complement to disrupt cell membranes through the membrane attack complex of the assays that specifically quantitate complement activation products. Historically, complement activation products have often been referred to as "split products or "cleavage products" because they are enzymatically cleaved from the parent proteins (e.g. C4 cleaves into C4a and C4d); however, it is recommended that these terms be avoided because some activation products are actually complexes that are formed during complement activation (e.g. C1rs-C1 inhibitor and SC5b-9) (Kirschfink and Mollnes 2003; Bergseth et al. 2013).

The most common functional assay used to assess activation of the classical pathway is termed the CH50 test. The principle of this assay is that by incubating test serum with immunoglobulin coated sheep erythrocytes in a buffer providing free Ca++ and Mg++, the degree of hemolysis (as measured by the amount of hemoglobin released) that occurs is directly proportional to the magnitude of complement activation. The results are typically expressed as the reciprocal dilution of the serum that is required to lyse 50% of a pre-determined amount of coated sheep erythrocytes. A similar assay, termed AH50, has been established for functionally evaluating the alternative pathway through addition of rabbit or guinea pig erythrocytes in a Mg++EDTA buffer.

The advantages of these functional assays are the ability to evaluate the function of the entire pathway from initial activation through the action of the membrane attack complex and the flexibility of use in many different animal species. Potential drawbacks to this methodology include a high potential for variability due to inadequate sample handling, and the lack of sensitivity. Specific recommendations for preparing the serum to be used in functional complement assays have been described previously (Lachmann 2010). Serum is always preferable to plasma, and clotting should take place at room temperature. Prior to centrifugation, time should be allowed for the sample to clot completely, but should not exceed 1 h. Ideally, centrifugation should be a two-step process performed at room temperature. The initial centrifugation, performed at approximately 3000 × *g* for 5–10 min, is followed by a second centrifugation of the serum at approximately 20,000 × *g* for 2–5 min to remove all fragments. This serum is then aliquotted and frozen for future use at −80 °C (−20 °C is not recommended). Thawing of aliquots should be performed rapidly in a 37 °C water bath (Lachmann 2010).

Both the intact complement proteins and the activation products can be measured by immunochemical methods; however, in many cases the concentration of the parent protein is too high to detect significant decreases through complement activation. Complement activation is typically assessed through immunochemical quantitation of complement activation products, and the most common methods include enzyme immunoassays, radial immunodiffusion and electroimmunodiffusion (Oppermann and Wurzner 2010). The earliest assays for evaluating complement activation were enzyme immunoassays for the anaphylatoxicins C3a, C4a, and C5a. One challenge in measuring these products is their very short in vivo half-lives. Many other assays have been subsequently developed that quantitate factors that circulate for longer periods of time (e.g. C3dg, C4d, Ba and Bb); however, with the exception of Bb in non-human primates, these newer assays have not been validated or widely used in preclinical safety studies (Oppermann and Wurzner 2010).

In the setting of preclinical safety, complement is typically assessed in cynomolgus monkeys, and the most commonly measured activation products include C3a, C5b-9 and Bb. As with functional assays, sample handling is very important for accurate immunochemical quantitation. EDTA plasma is recommended as it inhibits C3 and terminal pathway activation more efficiently than citrate and heparin (Harboe et al. 2011; Lachmann 2010; Mollnes et al. 1988). Ideally, the plasma should be snap frozen and stored at −70 °C if it cannot be analyzed immediately after sampling (Bergseth et al. 2013). Storage of EDTA plasma at −70 °C for up to three years without significant activation has been reported (Harboe et al. 2011). Pfeifer et al. (1999) have also recommended adding nafamostat mesylate to EDTA tubes for improved complement inhibition in vitro (Pfeifer et al. 1999).

Mouse complement is notoriously more difficult to assess functionally than other species. This is due in part to marked strain differences and substantially higher activity in males than females (Lachmann 2010). The mouse classical pathway is quite unstable and does not tolerate freezing, while the alternative pathway is more stable and amenable to frozen storage. Reagents and kits for immunochemical quantitation of complement activation products for all pathways are readily available as the mouse is an important model for evaluating complement function (Kotimaa et al. 2015). To date, use of complements is very limited in dogs; only CH50 and C3 endpoints have been evaluated (Lebrec et al. 2012).

9.2.9 Cytokines

Cytokines are small molecular weight proteins or peptides produced by many cell types to produce a biological response. In the immune system, cytokines regulate the duration and intensity of the immune response and are often key contributors to immunotoxicity, including both immunosuppression and immune stimulation (Corsini and House 2010). Cytokine activation can be analyzed by quantitating either mRNA or protein, depending on the specific goals of the study.

In the setting of most clinical pathology laboratories, cytokines are measured at the protein level by single-plex or multi-plexed ELISAs. To date, two of the most commonly used multiplex platforms are an electrochemiluminescent plate detection method (Meso Scale Diagnostics; Rockville, Maryland) and a fluorescent bead platform (Luminex) (Tarrant 2010). Both technologies claim a high degree of sensitivity, low background, flexibility and a wide dynamic range. A study performed by (Chowdhury et al. 2009) concluded superior sensitivity and accuracy with the MSD assay and superior precision with the Luminex assay. One of the most common challenges of multiplexed immunoassays is minimization of lot-to-lot variation. This problem has been a particular challenge for the MSD platform; however, recent advances in their electrochemiluminescent technology (V-Plex) have been developed to address the FDA's analytical validation guidelines and minimize lot-to-lot variation. Quantitation of mRNA is typically performed in a specialized molecular biology diagnostic laboratory.

One of the most important factors to consider when measuring cytokines from serum or plasma is the circulating half-life, which may be very short (minutes). Knowledge of the time at which cytokine levels peak following administration of a test item can be very important in obtaining cytokine levels that provide meaningful information on alterations in an immunologic response (Corsini and House 2010). Although multiplexed assays are clearly a major advancement in the ability to measure a large number of cytokines from a single, small volume sample, consideration must be given to the fact that a single timepoint may not represent the optimal time to evaluate each cytokine in a multi-plexed panel. Consequently, the multi-plexed panel may need to be performed at multiple timepoints for optimal evaluation of all cytokines.

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Chapter 10
Application of Immunohistochemistry in Toxicologic Pathology of the Hematolymphoid System

Jerold E. Rehg and Jerrold M. Ward

Abstract Immunohistochemical analysis of formalin-fixed, paraffin-embedded (FFPE) tissues can evaluate the characteristics of immune cells in the same tissue that is routinely collected for toxicologic pathology assessment. This chapter will address which antigen markers are best used for assessing toxicologic effects in the hematolymphoid system and for diagnosing hematolymphoid neoplasms in FFPE tissues from mice and rats. These markers can be normally present on immune cells, which may increase or decrease in number or undergo a change in their level of marker expression after exposure to a toxin, toxicant, or immunomodulator.

Keywords Immunohistochemistry • Hematolymphoid system • Rodent • Toxicology

10.1 Introduction

Immunohistochemical analysis of formalin-fixed, paraffin-embedded (FFPE) tissues can be used to evaluate the characteristics of immune cells in the same tissue that is routinely collected for toxologic pathology assessment. For this approach, the tissue specimens do not require special handling, as may be necessary with other techniques, such as flow cytometry to identify cell types in tissues; cytologic and architectural features are well preserved, allowing for improved histologic interpretation and comparisons with antigen localization in normal tissues and lesions.

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Modern immunohistochemical studies of paraffin tissue sections make it possible to do the following:

1. Evaluate and determine the normal and abnormal expression patterns of antigens in cells and tissues.
2. Determine whether cellular antigens are lost.
3. Identify alterations in the number or distribution of hematolymphoid cells in a tissue.
4. Detect changes in the cell surface markers of immune cells in response to inflammation or immunodulation.
5. Identify the types of immune and other cells associated with an inflammatory lesion.
6. Easily detect toxic effects, such as apoptosis.
7. Diagnose hematopoietic disorders, such as autoimmunity or neoplasms.

Unfortunately, most hematolymphoid antigen markers are not cell-lineage specific. Consequently, panels of antibodies are needed to confirm the lineage of a cell, and the makeup of this panel will depend on the cellular lineage in question.

Several reviews of the use of antibodies to detect cellular antigens of the myeloid and lymphoid lineages in FFPE tissues of rodents have been published (Ward et al., 2006, Rehg et al., 2012, Rehg et al., 2015). Here, we provide an updated and expanded compilation of our previous publications (Rehg et al., 2012, Rehg et al., 2015) and examples of how immunohistochemical analysis can be used to address specific research questions. Our sources of antibodies are listed in Tables 10.1, 10.2, 10.3, and 10.4, but other commercially available antibodies may work equally well.

10.2 Technical Considerations

Several excellent reviews, books, and websites address the techniques and development of rodent immunohistochemical analysis (Shi et al., 2011, Mikaelian et al., 2004, Ramos-Vara and Miller, 2014, Ward and Rehg, 2014); ((NCI, 2016), (NIEHS, 2016), The Jackson Laboratory, http://tumor.informatics.jax.org/html/antibodies.html). Here, we provide a brief synopsis of relevant topics that need to be considered when preparing to conduct an immunohistochemical analysis.

10.2.1 Tissue Preparation and Fixation

High-quality immunohistochemical analysis begins with tissue collection. The tissue specimens should be no thicker than a nickel (2 mm) and no larger than a postage stamp (2 cm), and they should be placed into the fixative immediately. Delayed fixation can lead to autolysis and a dried-out tissue specimen. Proteolysis as a result of autolysis can cause an increase in background staining, a decrease in antigen

Table 10.1 Antibodies used for mouse lymphoid cell antigens in immunotoxicity studies[a]

Antigen	Major cells expressing antigen	Antibody[b]	Clone[c]	Dilution	Source	Epitope retrieval
BCL2	Non–germinal center B cells; T cells; many lymphomas except mouse FL	RbAM	n/a	1000	BD Biosciences	HIER, (pH 9)
BCL6	Germinal center (centroblasts and centrocytes) B cells; GC or post-GC B-cell neoplasms	RAH	n/a	100	Santa Cruz Biotechnology	HIER, (pH 6)
CD3	T cells and T-cell neoplasms	GAM	n/a	350	Santa Cruz Biotechnology	HIER, (pH 6)
CD4	T-helper cells; Langerhans cells, some dendritic cells	RAM	4SM95	40	eBioscience	HIER, ER2 (pH 9)
CD5	T cells and T-cell lymphomas.	RAM	53-7.3	200	BD Biosciences	HIER, (pH 6)
CD8	Cytotoxic T-cells, NK cell subsets, dendritic cells	RAM	4SM15	50	eBioscience	HIER, ER1 (pH 6)
CD25	Activated T and B cells; some progenitor T & B cell stages, T-regs, immature myeloid cells	RbAM	SP176	30	Spring Bioscience	HIER, (pH 6)
CD43	All hematolymphoid cells; pro-B–lymphoblastic lymphoma, T-cell lymphomas, plasma cell neoplasms, myeloid leukemia	RAM	S7	20	BD Biosciences	HIER, (pH 6)
CD44	Subsets of T progenitors, activated T cells, monocytes and granulocytes.	RAM	IM7	40	BD Biosciences	HIER, CC1 (pH 8)
CD45	All hematolymphoid cells except erythroid cells	RAM	30-F11	150	BD Biosciences	HIER, (pH 6)
CD45R/B220	B cells, subsets of T cells, dendritic cells, and NK cell subsets	RAM	RA3-6B2	4000	BD Biosciences	HIER, Citrate (pH 6)
CD79acy	All B cells, Plasma cells; B lymphomas and plasmacytoma	MAH	HM57	50	Dako	HIER, (pH 9)
CD90.1	Immature T cells stronger than mature cells.	MAR	HIS51	50	BD Biosciences	HIER, (pH 9)
CD90.2	Immature T cells stronger than mature cells.	RAM	30-H12	30	Novus	HIER, Citrate (pH 6)
CD117/KIT	Immature B and T cells	RAH	n/a	100	Dako	HIER, (pH 6)
CD127/IL7Rα	Immature B cells; double-neg T cells	RbAM	Mfa	125	LifeSpan BioSciences	None

(continued)

Table 10.1 (continued)

Antigen	Major cells expressing antigen	Antibody[b]	Clone[c]	Dilution	Source	Epitope retrieval
CD138	Immature B cells, plasma cells	RAM	281-2	1000	BD Biosciences	HIER, ER1 (pH 6)
Foxp3	CD4+CD25+ regulatory T cells	RAM	FJK-16 s	100	eBioscience	HIER, (pH 6)
Granzyme B	Natural killer cells, NK T cells, cytotoxic T cells and mast cells	RAH	n/a	1000	Abcam	HIER, ER2 (pH 9)
IRF4/MUM1	Normal and neoplastic plasma cells. Post-GC cells and their neoplasms.	GAM	n/a	6000	Santa Cruz Biotechnology	HIER, (pH 6)
IgM	Immunoglobulin heavy chains of some immature B-cells; mature B cells; plasma cells	RAM	II/41	60	BD Biosciences	HIER, (pH 6)
KLC	Immunoglobulin light chains of most mature B cells; plasma cells	GAM	n/a	2000	Southern Biotechnology	HIER, (pH 6)
PAX5	B cells; B lymphomas; not expressed in plasma cells	GAM	n/a	300	Santa Cruz Biotechnology	HIER, Citrate (pH 6)
Perforin	Natural killer cells; NK-T cells, cytotoxic T cells	RAM	CB5.4	1000	Abcam	HIER, ER2 (pH 9)
PNA Lectin	GC cells & FDC	n/a	n/a	200	Vector	HIER, Citrate (pH 6)
RUNX1/AML	Myeloid cells, megakaryocytes, and lymphoid cells	RAH	n/a	900	Active Motif	HIER, ER1 (pH 6)
TDT	Immature thymic lymphoid cells, lymphoblastic neoplasms	RAH	n/a	50	Supertechs	HIER, Citrate (pH 6)

[a]Adapted from Rehg et al., 2012

[b]Abbreviations: *GAM* goat anti-mouse, *MAH* mouse anti-human, *MAR* mouse anti-rat, *RAM* rat anti-mouse, *RAH* rabbit anti-human, *RbAM* rabbit anti-mouse

[c]n/a, not applicable

Table 10.2 Antibodies used for mouse myeloid cell antigens in immunotoxicity studies[a]

Antigen	Major cells expressing antigen	Antibody[b]	Clone[c]	Dilution	Source	Epitope retrieval
CD34	Progenitor cells, some myeloid and lymphoblastic neoplasms	RAM	RAM34	50	BD Biosciences	HIER, (pH 6)
CD41	Megakaryocytes, platelets, early myeloid progenitors	RAM	n/a	200	Novus	HIER, Tris-EDTA (pH 9)
CD68	Histiocytes, myeloid cells, mast cells, and their respective neoplasms	RAM	FA-11	50	AbD Serotec	HIER, (pH 6)
CD117/Kit	Immature myeloid, T, and B cells and mast cells	RAH	n/a	100	Dako	HIER, (pH 6)
GATA 1	Erythroid cells, megakaryocytes, eosinophils, basophils, and mast cells	GAM	n/a	500	Santa Cruz Biotechnology	HIER, (pH 6)
Glycophorin A	Immature, mature, and leukemic erythroid cells	GAM	n/a	40	Santa Cruz Biotechnology	HIER, EDTA (pH 8)
Granzyme B	Mast cells, natural killer cells, NK-T cells and cytotoxic T cells	RAH	n/a	1000	Abcam	HIER, ER2 (pH 9)
Histamine	Normal and neoplastic mast cells	RAH	n/a	6000	Fitzgerald	HIER, (pH 9)
Lysozyme	Histiocytes/monocytes and myeloid cells (benign and malignant)	RAH	n/a	3000	Dako	HIER, Citrate (pH 6)
MBP eosinophil	Eosinophils	RAM	MT-14.7	1000	Mayo Clinic Lee Lab	EIER, Proteinase K
MPO	Benign and neoplastic neutrophils and eosinophils	RAH	n/a	1500	Dako	HIER, Citrate (pH 6)
Perforin	Mast cells, natural killer cells, NK-T cells, and cytotoxic T cells	RAM	CB5.4	1000	Abcam	HIER, ER2 (pH 9)
RUNX1/AML	Myeloid cells, megakaryocytes	RAH	n/a	900	Active Motif	HIER, ER1 (pH 6)
TER 119	Immature, mature erythroid cells; erythroid leukemia cells	RAM	TER-119	30	BD Biosciences	No retrieval
Von Willebrand factor	Normal and neoplastic megakaryocytes, platelets	RAH	n/a	500	Dako	HIER, Citrate (pH 6)

[a]Adapted from Rehg et al., 2012
[b]Abbreviations: *GAM* goat anti-mouse, *RAH* rabbit anti-human, *RAM* rat anti-mouse
[c]*n/a*, not applicable

Table 10.3 Antibodies used for mouse dendritic cell and macrophage antigens in immunotoxicity studies in mice[a]

Antigen	Major cells expressing antigen	Antibody[b]	Clone[c]	Dilution	Source	Epitope retrieval
CD21/35	Follicular B cells (weakly); follicular dendritic cells	RAM	7G6	20	BD Biosciences	HIER, (pH 6)
CD40	Activated T cells, interdigitating dendritic cells, and some B cells	GAM	n/a	50	Santa Cruz Biotechnology	HIER, Citrate (pH 6)
CD68	Histiocytes, myeloid cells, mast cells, dendritic cells, and their neoplasms	RAM	FA-11	50	AbD Serotec	HIER, (pH 6)
CD163	Histiocytes/macrophages, histiocytic sarcomas	RbAM	n/a	100	Santa Cruz Biotechnology	HIER, (pH 6)
F4/80	Histiocytes/macrophages	RAM	BM8	500	Caltag	HIER, (pH 6)
IBA-1	Histiocytes/macrophages	RAH	n/a	500	Biocare Medical	HIER, Citrate (pH 6)
iNOS2	Activated neutrophils; Th1-activated macrophages	RAH	n/a	400	Santa Cruz Biotechnology	HIER, Citrate (pH 6)
Langerin	Langerhans cells; dendritic cell subsets	RbAM	n/a	500	Thermo Fisher	HIER, CC1 (Roche pH 8)
Lysozyme	Histiocytes/monocytes and myeloid cells (benign and neoplastic)	RAH	n/a	3000	DAKO	HIER, Citrate (pH 6)
MAC2	Histiocytes/macrophages	RAM	M3/38	20,000	Accurate	HIER, Citrate (pH 6)
MAC3	Histiocytes/macrophages	RAM	M3/84	100	BD Biosciences	HIER, Citrate (pH 6)
S100	Interdigitating dendritic cells, Langerhans cells, some histiocytes/ macrophages	RAC	n/a	1000	DAKO	HIER, Citrate (pH 6)
YM1 (CHI3L3)	Immature neutrophils; Th2-activated macrophages	GAM	n/a	1500	R&D	HIER, (pH 6)

[a]Adapted from Rehg et al., 2012

[b]Abbreviations: *GAM* goat anti-mouse, *RAC* rabbit anti-cow brain, *RAM* rat anti-mouse, *RAH* rabbit anti-human, *RbAM* rabbit anti-mouse

[c]*n/a*, not applicable

Table 10.4 Antibodies for detection of rat hematolymphoid antigens in paraffin and frozen tissues in immunotoxicity studies[a]

Antibody	Primary cell type that expresses the antigen	Antibody clone	Source	Catalog No.
CD3	All T cells	Polyclonal	Santa Cruz	SC-1127
CD4	T-helper cells	W3/25	AbD Serotec	MCA55G
CD5	Subset of T lymphocytes, most thymocytes and NK cells	OX-19	AbD Serotec	MCA52G
CD8	Subset of T lymphocytes, most thymocytes and NK cells	OX-8	AbD Serotec	MCA48R
CD11b[b]	Most macrophages, Kupffer cells, dendritic cells, granulocytes	OX-42	AbD Serotec	MCA275GA
CD25	Activated rat T cells	OX-39	AbD Serotec	MCA273R
CD41	Megakaryocytes	Polyclonal	NOVUS	NBP1-84,579
CD43	All leukocytes with the exception of mature B lymphocytes	W3/13	AbD Serotec	MCA54G
CD45RA	B cells among thoracic duct lymphocytes, with little labeling in bone marrow and none on thymocytes	OX-33	AbD Serotec	MCA340GA
CD45RC	B-cells among thoracic duct lymphocytes, with little labeling in bone marrow and none on thymocytes	OX-22	AbD Serotec	MCA53R
CD68	Myeloid cells	ED1	AbD Serotec	MCA341R
CD163	Most macrophages	ED2	AbD Serotec	MCA342GA
CD169[b]	Macrophages	ED3	AbD Serotec	MCA343R
IBA1	Macrophages	Polyclonal	Biocare Medical	CP290A
IgM	B cells	Polyclonal	Vector	BA-2020
IRF4	Plasma cells, post–germinal center B cells	Polyclonal	Santa Cruz	SC-6059
Kappa light chains (human)	Immunoglobulin-producing B cells	Polyclonal	Dako	A0191
Lysozyme	Macrophages and myeloid cells benign and malignant	Polyclonal	Dako	A0099
MHC Class II	B cells	OX-6	AbD Serotec	MCA46G
Myeloperoxidase (MPO)	Neutrophils, eosinophils, and their neoplasms	Polyclonal	Dako	A0398
PAX5	All B cells and B-cell lymphomas	Polyclonal	Santa Cruz	SC-1974
TDT	Immature B and T cells; B- and T-lymphoblastic lymphoma.	Polyclonal	Supertechs	004

[a]Adapted from Rehg et al., 2012.
[b]This antibody works only with frozen tissue.

detection, and the diffusion of soluble proteins. Dried-out tissue can result in an immunohistochemistry (IHC) edge effect. All of these artifacts can affect the interpretation of the immunoreactivity of a stained specimen.

10.2.2 Fixative and Fixation Time

No single fixative is ideal for all markers, antibodies, or applications (Grizzle, 2009). Therefore, the immunohistochemical assessments in this chapter are based on using formalin as the fixative, which has several advantages: formalin reliably provides good histologic quality; it is inexpensive; its deleterious effects on antigen recognition can generally be reversed with antigen retrieval, and DNA/RNA studies can be conducted on the FFPE tissue specimen.

There is no optimal fixation time for every antigen; both under-fixation and over-fixation can have deleterious effects on immunoreactivity. In immunohistochemical analysis, under-fixation is more problematic than over-fixation, because the significant reduction of immunoreactivity associated with over-fixation is problematic for only a few antigens and only after several weeks of fixation (Julie Randolph, Fred Hutchinson Cancer Research Center, personal communication; Webster et al., 2009). In most cases, the negative effects of over-fixation can be overcome with appropriate antigen-retrieval methods. Generally, complete fixation of a tissue with formalin is achieved in 24 to 48 h. The Fred Hutchinson Cancer Center Immunohistology laboratory reports that there is no significant difference in the reproducible immunohistochemical results obtained after formalin fixations of 3–12 days' duration, and others have also reported that immunohistochemical reactivity remained moderate to strong after up to 7 weeks of formalin fixation (Webster et al., 2009).

10.2.3 Decalcification

Decalcification with strong acid solutions negatively affects the immunoreactivity of some antigens (Ewing-Finchem, 2008). However, for most antigens, decalcification with weak acids does not appear to interfere significantly with immunohistochemical analysis, provided the tissues are well fixed in formalin (Ramos-Vara, 2005).

10.2.4 Immunohistochemistry Fundamentals

The specificity of a primary antibody is a key element in any immunohistochemical assay, but all antibodies are not equal. Western blot (WB) immunoreactivity with an antigen does not necessarily predict immunoreactivity in FFPE tissues.

Monoclonal antibody clones prepared to the same antigen may not produce the same immunohistochemical result, and different clones may not react with the same epitope (Rehg et al., 2012). It has also been reported that antibody to the same clone from different companies may yield different outcomes, suggesting that nonactive ingredients of the monoclonal preparation can interfere with the immunologic reaction (Ramos-Vara and Miller, 2014). We ourselves found that a new lot of rabbit anti-CD3 antibody obtained from the same supplier as previous lots unexpectedly stained Kupffer cells in normal mouse liver (Ward and Rehg, 2014). In view of these experiences, antibody specificity is best documented by the appropriate use of controls.

10.2.5 Specific Staining

Antigens may be present in the cell membrane, cytoplasm, and/or the nucleus or extracellular areas (Ward and Rehg, 2014). The nucleoplasm and nuclear membrane may be positive by immunohistochemical staining. The cell cytoplasm or specific organelles (mitochondria, lysosomes, rough and smooth endoplasmic reticulum, or peroxisomes), lipid droplets, intermediate filaments, pigment granules, or induced abnormal structures can also serve as detectable antigens.

Outside the cell, connective tissue, tissue matrices, extracellular fluids, necrotic debris, blood components, and outer substances both normal and abnormal can be immunoreactive. An antigen may be found in a particular cellular location in one cell type and in a different location in another cell type. A genetic mutation can result in a shift in the location of an antigen; for example, a cell membrane or cytoplasmic antigen may be found in the nucleus after such a mutation. Consequently, it is essential to determine where a target antigen is expected to be located.

10.2.6 Controls

It is essential that a positive and negative antigen tissue sample be used for each run of an immunohistochemical assay. A positive tissue control is a tissue in which the targeted antigen is known to be detectable by immunohistochemical methods identical to those used on the test sample. An external positive tissue control serves to assess the performance of the primary antibody, whereas an internal control assesses whether the immunoreactivity of the test antigen within the test specimen and/or on the same slide is adequately preserved for immunohistochemical analysis. Positive control tissue should be obtained and processed by the immunohistochemistry (IHC) laboratory, because the fixation and processing procedures at other laboratories may be different. A negative tissue control is a tissue that is known not to contain the antigen of interest. The negative control tissue should be processed in the

same manner as the test sample and the positive control tissue. Nonspecific controls without the primary antibody are used to confirm the test specificity and to assess the degree of nonspecific background staining caused by the secondary antibody. The control is commonly conducted by replacing the primary antibody with (1) same-species nonimmune immunoglobulin at the same concentration as the primary antibody, (2) an irrelevant antibody, or (3) a buffer. Of these three alternatives, the latter is the least preferred.

The number of antibodies available to characterize hematolymphoid cells in mice and rats has increased significantly over the past 10 years. The major antibodies that are useful for immunohistochemical analysis of FFPE mouse and rat tissues are listed in Tables 10.1, 10.2, 10.3, and 10.4 (Rehg et al., 2012, Rehg et al., 2015).

10.2.7 Antibodies and Antigens for FFPE Tissues

The next section lists and discusses a series of antibodies and antigens that are used in the immunohistochemical analysis of several species, but the text is directed towards their use in mouse studies. Rat, monkey, dog, and pig lymphoid IHC is discussed at the end of this section. Several antibodies will work in all species, but most are species specific, especially monoclonal antibodies. References are given for the major antibodies used for each species.

10.3 Panleukocyte Antigen

The CD45 antigen (the leukocyte common antigen, encoded by the *Ptprc* gene) is a family of protein tyrosine phosphatases that are expressed on virtually all hematolymphoid cells and their progenitors, with the exception of maturing erythroid cells and megakaryocytes. The antigen is not commonly expressed on plasma cells and is not present on any nonhematopoietic cells (Weiss et al., 1993). Positive immunohistochemical staining for CD45 is localized to membranes, but occasional paranuclear staining is observed. The anti-CD45 antibody labels all isoforms of the family, whereas anti-CD45R antibodies are reactive only with an epitope of CD45 and are thus restricted to binding subsets of the CD45 family of proteins.

10.4 B-Cell and Plasma Cell Lineage Antigens

10.4.1 B-Cell Antigens

Mouse precursor B cells arise from the bone marrow and then migrate to secondary lymphoid tissue, such as the lymph nodes, spleen, and mucosa-associated lymphoid tissue.

Table 10.5 IHC detectable antigens associated with mouse B-cell differentiation[a,b]

	Pro-B cell			Pre-B cell				
	Early	INT	Late	Large	Small	Immature	Mature	Plasma Cell
CD117	xxxxxxxxx. . . . . [c]							
TdT	xxxxxxxxx.							
CD43	. . . . .							xxxxxxxxxxx
CD25			. . . .	xxxxxxxxxxxxxxxxxx				
CD127	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx.							
CD138	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							xxxxxxxxxxx
PAX5	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							
CD45R/B220	. . . . .			xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx				
$_{c}$μ heavy chains[d]				xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx				
$_{s}$IgM[e]						xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx[f]		
Ig light chains						. . . . .	xxxxxxxxxxxxxxxxxxxxxxxx	

[a] This schematic is not intended to be comprehensive, and expression can be altered as a result of cellular environment, differentiation state and other factors

[b] Table is a composite based on data from multiple sources (Osmond et al. 1998; Ceredig and Rolink 2002; Hardy et al. 2007; Rehg et al. 2012)

[c] x indicates high to moderate expression; … indicates low/weak expression

[d] $_{c}$μ, cytoplasmic μ

[e] $_{s}$Ig, surface immunoglobulin

[f] Only some plasma cells express IgM and it is generally cytoplasmic

Early B cells, called precursors, progress sequentially through several developmental stages: pro-B cell to pre-B cell to naïve immature B cell. Following migration from the bone marrow, the naïve immature B cells migrate to the lymphoid organs where they further differentiate and mature into memory B-cells or plasma cells, usually upon migration through the germinal center. The B cell–related antigens expressed in the different stages of differentiation also change (Table 10.5). During B-cell ontogeny, some proteins control B-cell differentiation or function as B cell–specific receptors. These proteins include PAX5 and CD79a.

The CD43 antigen, also known as leukosialin and sialophorin (encoded by the *Spn* gene), is non–lineage specific and is expressed on most granulocytes, immature and mature T lymphocytes and their progenitors, pro-B lymphocytes, plasma cells, and their neoplastic counterparts (Arber and Weiss, 1993, Rehg and Sundberg, 2008, Rehg et al., 2015). It is also observed on the plasma cells of lymphomas with plasma cell differentiation. In the mouse, non-neoplastic mature B lymphocytes are CD43 negative.

The antigen is usually expressed on cell membranes, but cells may exhibit both membranous and diffuse cytoplasmic staining in an immunohistochemical assay (Arber and Weiss, 1993, Rehg et al., 2012). It should be a component of biomarker panels for detecting normal and neoplastic myeloid cells, immature B cells, T cells, and plasma cells. In addition to the effect of insufficient fixation on the immunoreactivity of CD43, the authors have also noticed that decalcification with Cal Rite may, in some instances, diminish the immunoreactivity of CD43 in bone marrow cells. We have also noticed that plasma cells appear to be more sensitive than T lymphocytes

to a narrow range of heat retrieval temperatures. Therefore, it is recommended that a tissue with plasma cells be used to optimize the CD43 immunohistochemical assay.

CD45R/B220 is a cell membrane antigen that is recognized as a pan–B-cell antigen (it is a different epitope to CD45 but is encoded by the same gene, *Ptprc)*. Flow cytometry studies have demonstrated that this antigen is expressed at low levels at the pro-B cell stage, but the intensity level at this stage is often too low to be detected by immunohistochemical staining (Rehg and Sundberg, 2008, Rehg et al., 2015). CD45R/B220 antigen expression increases in intensity as B-cell differentiation progresses (Osmond et al., 1998, Hardy et al., 2007), but the expression ceases before the cells differentiate into plasma cells. In addition to being undetectable by immunohistochemical staining in pro-B cells and plasma cells, CD45R/B220 expression is undetectable in 20% of marginal zone lymphomas (Kunder et al., 2007, Rehg and Sundberg, 2008). T cells are essentially negative for CD45R/B220, except for certain subsets of T cells and T-cell lymphomas that occur in some genetically engineered mice (Kunder et al., 2007, Rehg and Sundberg, 2008). The CD45R/B220 antigen is also expressed in a subset of NK cells (Fig. 10.1) (Blasius et al., 2007, Vosshenrich et al., 2007), and plasmacytoid dendritic cells (Shortman and Liu, 2002, Haniffa et al., 2013).

CD79a (encoded by the *Cd79a* gene) is an intracellular component of the B-cell receptor. Weak CD79a expression occurs in the pre-B cell stage, and expression intensifies and persists throughout the various stages of B-cell differentiation. CD79 is more strongly expressed in follicle, mantle, and marginal zone B cells than in germinal center B cells, suggesting that the activation of GC cells into memory or plasma cells downregulates CD79a expression. The immunoreactive staining pattern is cytoplasmic and may be weak or undetectable in immature B cells, germinal center cells, and plasma cells (Rehg et al., 2012). The anti-CD79acy antibody recognizes the CD79a antigen, and normal mouse T cells are not immunoreactive. It remains to be determined whether CD79a is expressed in any mouse myeloid leukemias or T-lymphoblastic lymphomas but such expression has been reported in humans (Pilozzi et al., 1998, Bhargava et al., 2007).

The *IRF4/MUM1* (interferon regulator factor 4/multiple myeloma oncogene 1) gene encodes a transcription factor that plays a key role in the development of lymphoid cells. The IRF4/MUM1 protein is primarily expressed in B cells, plasma cells, and activated T-lymphoid cells, with the highest levels being expressed in plasma cells and activated T cells (Gualco et al., 2010). IRF4/MUM1 is expressed in a small subset of germinal center cells that are committed to plasmacytic or memory cell differentiation. Anti-IRF4/MUM1 antibody labels the nuclei and cytoplasm of cells expressing the antigen, with the intensity in the nucleus being greater than that in the cytoplasm. In addition to being expressed in plasma cells and myeloma, IRF4/MUM1 protein is expressed predominantly in human lymphoid neoplasms thought to be derived from late-stage follicular germinal center and post–germinal center B cells (Falini et al., 2000). The type of mouse lymphomas that express IRF4 remains to be characterized; however, the authors have found the IRF4/MUM1 antigen to be an excellent marker for plasma-cell neoplasms in mice (Rehg et al., 2012).

The *Pax5* gene is a member of the PAX family of transcription factors. PAX5 protein is essential for B-cell development and activation (Adams et al., 1992). PAX5

expression is nuclear and is detected in pro-B cells, pre-B cells, and immature and mature B cells, but its expression is down-regulated in plasma cells. PAX5 protein is detected in essentially all B-cell lymphomas/leukemias in mice (Kunder et al., 2007, Rehg and Sundberg, 2008, Rehg et al., 2012). There is good correlation between PAX5, CD45R/B220, and CD79a expression. However, for some lymphomas, PAX5 appears to be a more sensitive immunohistochemical marker than CD45R/B220 (Rehg and Sundberg, 2008, Rehg et al., 2015). The apparent greater sensitivity of PAX5, as compared to CD45R/B220, is more than likely associated with the low level of CD45R/B220 on pro-B cells and some marginal zone lymphomas and the inability of immunohistochemical staining to detect the low levels of CD45R/B220 on these cells. PAX5 is more specific than CD79a for diagnosing human B-lymphoblastic lymphoma and leukemia (Pilozzi et al., 1998), but a similar discrepancy has not yet been demonstrated in mice.

10.4.2 Immunoglobulins

Immunoglobulins are uniquely produced and expressed by B cells, including plasma cells, and are more reliably assessed by flow cytometry or by frozen-section immunohistochemical analysis. However, immunoglobulins may also be detected in paraffin sections, especially when expressed in the cytoplasm. B cells can express each of the heavy-chain classes, i.e., IgM, IgG, IgA, and IgE. One individual B cell can express more than one heavy-chain class. Immunoglobulins can be expressed in the cytoplasm or on the surface of cells, depending on the stage of cell maturation. Because some B-cell stages have scanty cytoplasm, immunohistochemical analysis cannot differentiate between cytoplasmic and surface immunoglobulin associated with these cells. Unlike that in humans, light-chain immunoglobulin expression in the mouse consists of 95% kappa light chain (Woloschak and Krco, 1987), but a polyclonal anti-human kappa light-chain antibody is good for detecting immunoglobulin in the mouse. The demonstration of μ heavy chains without light-chain expression in B lymphoblasts serves to distinguish pre-B lymphoblasts from pro-B lymphoblasts in normal development and in lymphoma/leukemia.

10.4.3 Plasma Cell Antigens

Plasma cell antigens are not specific to these cells. However, when used in context with a cell's cytologic morphology and lack of PAX5 and/or CD45R/B220 expression, antibodies to the CD43, CD138, and IRF4/MUM1 antigens are very sensitive and extremely useful in identifying plasma cells (Rehg et al., 2012). In the authors' experience, insufficient fixation affects the immunohistochemical detection of CD43 and CD138, and decalcification with Cal Rite may, in some instances, diminish the immunoreactivity of CD43.

10.5 T-, NK–T and NK Cell Lineage Antigens

T lymphocytes arise from the bone marrow then migrate to the thymus, where they differentiate into mature T cells. The differentiation process in the thymus is characterized by distinct and sequential patterns of antigen expression, as initially characterized by flow cytometry. Many of these antigens can now be detected by immunohistochemical staining. The thymocytes are categorized as double negative (DN), double positive (DP), or single positive (SP) based on their expression of CD4 and CD8 receptors. In mice, the thymocytes can be further subdivided into four subsets (Pro-T1, Pro-T2, Pre-T1, and Pre-T2) based on the expression of CD2, CD3, CD5, CD7, CD44, CD25, CD117, and TDT (Table 10.6) (Lesley et al., 1988, Godfrey et al., 1993). CD7 is the earliest expressed T-leukocyte antigen. This is followed by CD2 and CD5 expression along with cytoplasmic expression of CD3. Within the thymus, there is co-expression of CD4 and CD8 at the pre-T2 stage (i.e., the cells are DP), along with surface expression of CD3. Because the T cells at this stage have scanty cytoplasm, it is not feasible to differentiate cytoplasmic and

Table 10.6 Immunophenotype of mouse T-cells during development[a]

			Cortical thymus			Medullary thymus	Mature peripheral tissue
	Pro-T_1 (DN1)	Pro-T_2 (DN2)	Pre-T_1 (DN3)	Pre-T_2 (DN4)	DP[b]	SP	
CD4	–	–	–	–	+	+ or –	+ or –
CD8	–	–	–	–	+	+ or –	+ or –
CD44	++	+++	–	–	–	–	Activated cells +
CD25	–	+	++	–	–	–	Activated cells +
CD117 (Kit)	+++[c]	++	+	+ or -	–	–	–
CD127 (ILRα)	–	+++	++	+	–	–	–
CD7[d]	+++	+++	+++	+++	++	++	+ or –
CD3	C	C	C	C	C	S	S
CD2[d]	–	++	++	++	++	++	++
CD5	–	++	++	++	++	++	++
CD43	++	++	++	++	++	++	++
CD90	–	++	++	++	++	++	++
TDT	++	++	++	++	++	–	–

[a]Table is a composite of data based on flow cytometry and IHC from multiple sources (Ceredig and Rolink, 2002, Dik et al., 2005, Weerkamp et al., 2006, Bhandoola and Sambandam, 2006, Mazzucchelli and Durum, 2007, Rehg et al., 2012)

[b]Abbreviations: *C* cytoplasm, *S* surface, *DP* double positive, *SP* single positive

[c]Expression is semi quantitative grade; –, negative; +, low; ++, medium; +++, high

[d]Antigens are not detectable by immunohistochemical staining in FFPE tissue

surface CD3 expression by immunohistochemical staining. Upon exiting the thymus, the T cells express either CD4 or CD8, but not both. CD4-positive cells (helper T cells) outnumber CD8-positive cells by a ratio of 2:1 in lymphoid tissue. On occasion, a T-cell lymphoma may lack expression of one or more of these antigens.

With the availability of CD4, CD8, CD25, and CD44 antibodies for use in FFPE tissues, it should now be feasible to classify a T-lymphoblastic lymphoma according to whether it is a pro–T- or pre–T-lymphoblastic lymphoma and whether the tumor lymphoblasts are DN or DP.

CD2 is a pan–T-cell antigen (encoded by the gene *Cd2*) that is expressed on the membrane of immature and mature T and NK cells and most T-cell lymphomas. At present, there is no antibody to CD2 that can be used with FFPE mouse tissue.

CD3 is a lineage-specific protein (encoded by the *Cd3e* gene) that is considered to be a pan–T-cell marker. CD3 is expressed first in the cytoplasm of immature T cells and subsequently on the cell membrane of mature T lymphocytes. It may be difficult to distinguish between intracellular and cell-membrane expression of CD3 in T cells by immunohistochemical staining because of the cells' scant cytoplasm. Therefore, a CD3+ lymphoma should also be labeled with anti-TDT antibody to determine whether it is an immature or mature T-cell lymphoma; however, on rare occasions, a T-lymphoblastic lymphoma may not express TDT (Patel et al., 2012), Rehg, unpublished observations). NK-T cells also express the epsilon unit of CD3 in their cytoplasm. Consequently, a high percentage of NK-T cells will be labeled by the polyclonal anti-CD3 antibody, which contains the epsilon unit.

CD4 is a cell membrane antigen (encoded by the *Cd4* gene) that is present on T-helper cells (Fig. 10.2), monocytes, human macrophages, Langerhans cells, and other dendritic cells, but not on B cells or mouse macrophages (Crocker et al., 1987). CD4 is expressed more commonly than CD8 in T lymphoma/leukemia in humans, but the frequency of CD4 and CD8 expression in rodent T lymphomas/leukemias remains to be determined. Aberrant T-cell double positivity for CD4 and CD8 or double negativity for CD4 and CD8 in a mature T-cell proliferation is sug-

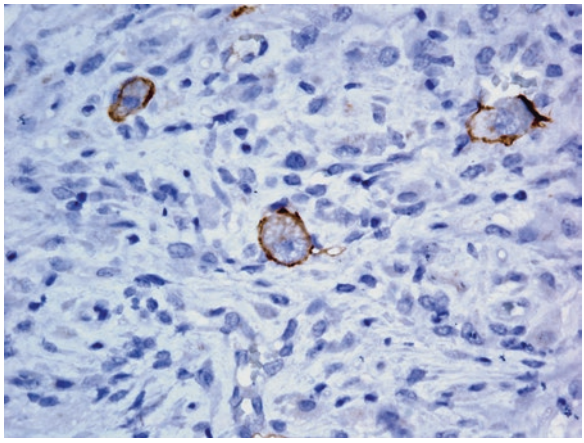

Fig. 10.1 A sclerotic lymph node from an irradiated C57BL/6 mouse showing cell membrane expression of CD45R/B220 on a few NK cells (Chromogen: DAB)

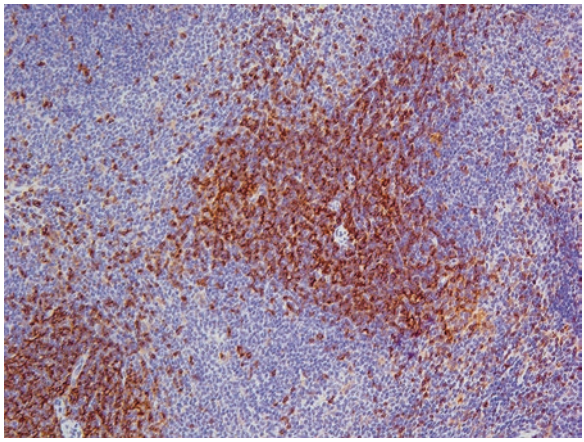

Fig. 10.2 Spleen of an adult FVB mouse showing cell membrane expression of CD4 on T lymphocytes in the periarteriolar lymphoid sheath (PALS) of the T zones (Chromogen: DAB)

gestive of a T-cell lymphoma. In autoimmune lymphoproliferative disorders in humans, the lymphoid cells commonly lack CD4 and CD8, but a similar absence has yet to be confirmed in rodents.

CD5 is a glycoprotein (encoded by the *Cd5* gene) that is expressed on the surface of most immature and mature T cells and NK-T cells. The antigen is not expressed on NK cells and is expressed on few—if any—intestinal intraepithelial lymphocytes bearing the γδ T-cell receptor. However, CD5 is expressed on the majority of T-lymphoblastic and mature T lymphomas (Kunder et al., 2007, Rehg et al., 2015). By using flow cytometry, CD5 has been reported to be detectable at a low level on all mouse lymphoid neoplasms except B-lymphoblastic lymphoma (Morse et al., 2002). However, this has not been the experience of the authors or other researchers when using immunohistochemical staining on B-cell neoplasms in the mouse (Kunder et al., 2007). The difference between these observations is probably explained by the level of CD5 expression on mouse B lymphocytes being too low to be detected by immunohistochemical staining but sufficiently high to be detected by the more sensitive flow cytometry method.

The CD8 cell membrane antigen (encoded by the *Cd8a* gene) is expressed on T cells with suppressor and cytotoxic activity and on a subset of NK cells. The antigen is also expressed in non-neoplastic and neoplastic splenic sinusoidal lining cells (littoral cells) of humans, but CD8 is not expressed on non-neoplastic mouse splenic sinusoidal lining cells (Fig. 10.3). Whether CD8 is expressed on neoplastic splenic sinusoidal lining cells in the mouse remains to be determined. The authors and others have noticed that the only commercial anti-CD8 antibody (eBioscience) that is available for use in FFPE mouse tissue cross-reacts with mouse brain neurons. The reason for this cross-reactivity is currently unknown. It should be noted that anti-PAX5 antibody reacts with some brain neurons and that this immunoreactivity is considered specific.

CD25 is the alpha chain of the IL-2 receptor. The CD25 antigen (encoded by the *Il2ra* gene) is present on the membrane of activated T cells, activated B cells, pro-T2/pre-T1 thymocytes, late pro-B/large pre-B cells, myeloid precursors, and macro-

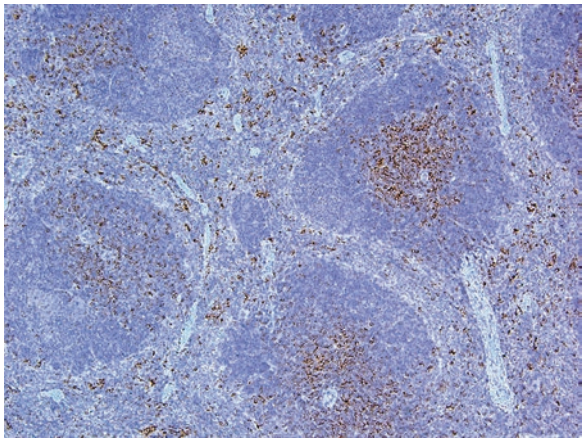

Fig. 10.3 Spleen of an adult FVB mouse showing the red pulp T lymphocytes with a random, nonsinusoidal distribution and the T lymphocytes in the white pulp PALS expressing CD8 (Chromogen: DAB)

phages. This antigen has been used as a marker for identifying T-cell developmental stages (Fig. 10.4) and CD4+ FoxP3+ regulatory T cells in mice. Resting T and B lymphocytes and NK cells do not express the CD25 protein. In humans, CD25 expression is a feature of mast cells associated with systemic mastocytosis (Horny et al., 2010).

CD44 is a cell surface antigen (encoded by the *Cd44* gene). In mice, it is expressed on specific subsets of immature T cells (Fig. 10.5), activated peripheral T lymphocytes, monocytes, and granulocytes. CD44, along with CD25, is used to track early T-cell development in the mouse thymus and to determine the stage of T-lymphoblastic lymphoma/leukemia. BALB/c T lymphocytes express a higher level of CD44 than do T lymphocytes of other mouse strains (Lynch and Ceredig, 1989). Germinal center cells do not express CD44 at all, but post–germinal center cells (PGCs) express high levels of CD44 (Kremmidiotis and Zola, 1995). Whether CD44 can be a marker for differentiating PGC lymphoma from follicular cell lymphoma in mice requires further investigation.

The CD90 antigen (encoded by the *Thy1* gene) is a conserved cell surface protein that was the first T-cell marker to be identified. It is present on thymocytes (immature thymic T cells that differentiate into mature peripheral T lymphocytes), mature peripheral T lymphocytes, and activated NK cells in the mouse and on human neuronal cells. In mice, there are two alleles: Thy1.1 (encoded by *Thy1a,* CD90.1) and Thy1.2 (encoded by *Thy1b*, CD90.2). These two alleles differ by only one amino acid at position 108; an arginine in CD 90.1 and a glutamine in CD90.2. CD90.1 is expressed by the AKR/J and PL mouse strains, whereas CD90.2 is expressed by most other mouse strains. Mouse cortical thymocytes express higher levels of CD90 than do medullary thymocytes (Fig. 10.6), which in turn express higher levels of CD90 than do lymph-node T cells. A similar inverse developmental temporal expression profile is seen in rat T cells, although rat CD90 is lost at an earlier stage of T-cell maturation (Crawford and Barton, 1986). Consequently, CD90 is only expressed on thymocytes in rats.

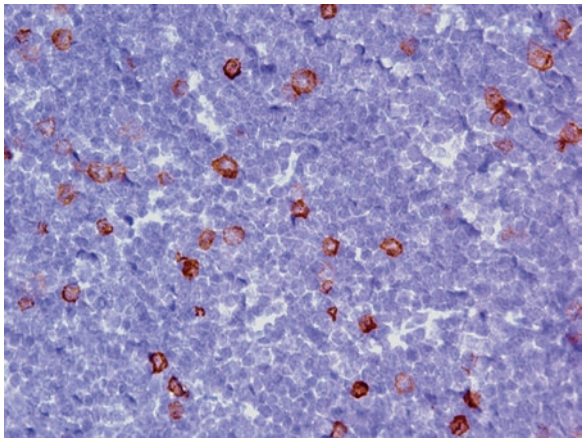

Fig. 10.4 Thymus of an adult FVB mouse showing cell membrane expression of CD25 on a few immature DN2 or DN3 cortical thymocytes (Chromogen: DAB)

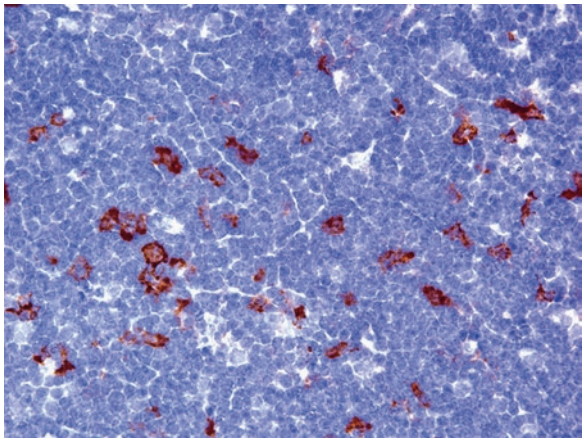

Fig. 10.5 Thymus of an adult FVB mouse showing cell membrane expression of CD44 on a few immature DN1 or DN2 cortical thymocytes (Chromogen: DAB)

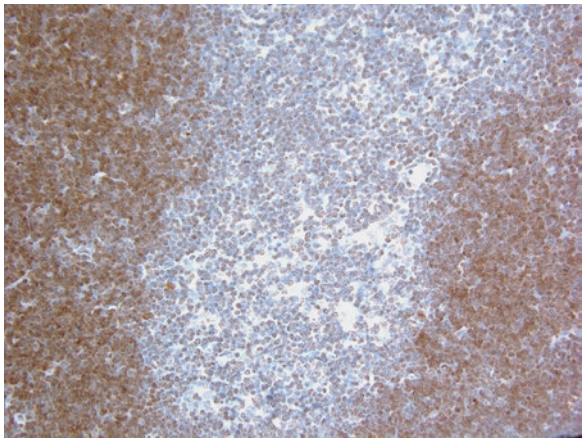

Fig. 10.6 Thymus of an adult FVB mouse showing cortical thymocytes with more intense membrane expression of CD90.2 than in medullary thymocytes (Chromogen: DAB)

FOXP3 (fork head box p3, also known as SCUFRIN and JM2) is a protein (encoded by the *Foxp3* gene) that is expressed on thymic CD4+CD25+ T-regulatory (T reg) cells and on some, but not all, inducible T reg cells.

Granzymes (granule enzymes) are a family of highly homologous serine proteases contained in the cytotoxic granules of innate and adaptive immune killer cells. The major function of these enzymes is to induce cell death in order to eliminate viruses and tumor cells. However, granzymes may also play a role in immune regulation and the regulation of inflammation. Granzyme A and granzyme B are the most abundant granzymes. To the authors' knowledge, there is no commercially available antibody to mouse granzyme A for use in FFPE tissue.

Granzyme B (encoded by the *Gzmb* gene) and perforin (encoded by the *Pfr1* gene) are two major toxic proteins located in the cytoplasmic granules of cytotoxic T cells and NK-T and NK cells, including the hepatic NK cells ("pit cells"). Granzyme B and perforin proteins have been considered to be constitutively expressed in human and mouse NK cells. However, NK cells in specific pathogen–free (SPF) mice contain mRNAs for granzyme B and perforin but none or only very little of the two enzymes. Upon activation of the SPF mouse NK cells, the cells express abundant levels of both proteins and are highly cytotoxic (Fehniger et al., 2007). It should be noted these two enzymes are also seen in large granular NK cell leukemia in F344 rats (Thomas et al., 2007). Whether Fehniger's observation in mice is also true for SPF rats remains to be investigated. Furthermore, the granules of eosinophils, mast cells, and human plasmacytoid dendritic cells also express granzyme B (Costain et al., 2001, Pardo et al., 2007). Whether mouse plasmacytoid dendritic cells express granzyme B as do their human counterparts remains to be determined.

Cytotoxic T cells, also known as killer T cells, are activated T lymphocytes that express surface CD3 and CD8 or CD4, cytoplasmic granzyme B, and/or perforin. Although some cytotoxic T cells are CD4 T cells, the majority are CD8 T cells.

NK-T cells in the mouse represent a distinct, heterogeneous sublineage of T lymphocytes that are restricted by a nonclassical MHC class I molecule (CD1d) that is associated with β2-microglobulin. Most NK-T cells express an invariant T-cell receptor (TCR) and are termed iNK-T cells. NK-T cells are proportionally more abundant in the liver (constituting 30–50% of liver lymphocytes), thymus (10–20%), blood (4%), spleen (3%), and lymph nodes (< 1%) (Laloux et al., 2002). NK-T cells share a number of surface markers with NK cells. Some cells express CD49b and some express NK1.1 (CD161), depending on their state of maturity and activation, the tissue location, and the mouse strain (Kronenberg and Gapin, 2002). Unlike other T cells, NK-T cells are negative for surface CD3, but they are positive for cytoplasmic CD3ε and express CD5 and CD43 (Takeda et al., 2000). NK-T cells are usually positive for cytotoxic molecules, such as granzyme B and perforin, but only one or the other protein may be expressed (Kawamura et al., 1998). Some mouse NK-T cells may be doubly negative for CD4 and CD8, whereas others may express either CD4 or CD8.

NK cells are a specific subset of lymphocytes that lack T-cell receptors and B-cell receptors and do not require the target cell to express MHC class II. They have a cytotoxic function similar to that of NK-T cells and cytotoxic CD8 T lym-

phocytes. Unlike cytotoxic T cells and NK-T cells, mouse NK cells do not express surface or intracellular CD3, nor do they express CD5 (Stewart et al., 2007). Except for a small subset of NK cells, the vast majority of NK cells do not express CD4 or CD8, but mouse NK cells express CD11b, CD43, NK1.1 (CD161) or NK1.2 antigen, CD49b, granzyme B, and perforin. Until recently, the precept has been that human and mouse NK cells constitutively express the granzyme B and perforin enzymes. However, as indicated in the granzyme/perforin section above, resting (unactivated) NK cells in SPF mice cells contain mRNAs for granzyme B and perforin, but they either do not express the two proteins at all or express them at only a low level, unless the NK cells are activated (Fehniger et al., 2007). If this observation is confirmed, mouse cells that can be identified as NK cells by their surface markers but have not been activated may not be labeled by antibody to granzyme B or perforin. Furthermore, the NK1.1 and CD49b antigens are not expressed in immature NK cells (Stewart et al., 2007), and the NK1 antigens are mouse-strain restricted. NK1.1 is expressed in C57BL/6, FVB, and NZB mouse strains, whereas NK1.2 is expressed in BALB/c, 129, AKR, and C3H mouse strains. Mouse NK cells also express low levels of CD11c, and a subset of NK cells express CD45R/B220 (Fig. 10.1). Anti-CD49b (DX5) antibody was reportedly used to detect NK cells in FFPE mouse tissue (Kaufman et al., 2010), but this remains to be confirmed. A panel of antibodies to CD3, CD5, CD45R/B220, NK1, granzyme B, and perforin will aid in differentiating between mouse NK, NK-T, and cytotoxic T cells, as well as between B cells and plasmacytoid dendritic cells that express CD45R/B220. The CD45R/B220, NK1, and CD49b antigens are not suitable markers for identifying NK cells in the rat.

10.6 Other Lymphoid Antigens

The BCL6 antigen (encoded by the *Bcl6* gene) is expressed in normal follicular germinal center B cells (centroblasts and centrocytes) and in follicular (germinal center) and some post–germinal center neoplasms. By immunohistochemical staining, BCL-6 is localized in the nucleus with a microgranular or diffuse pattern (Rehg et al., 2012). BCL-6 protein expression does not correlate with BCL-6 gene rearrangement. BCL-6 is not expressed in marginal zone and mantle cells, plasma cells, or bone marrow precursor cells, nor is the antigen expressed in lymphomas of these respective cells. In the author's experience, the long-term storage of unstained slides can have a negative effect on BCL6 immunoreactivity that may lead to a false-negative result (King et al., 2000). Therefore, it is recommended that freshly cut paraffin sections be used for BCL6 immunohistochemical assays.

BCL-2 is a cytoplasmic anti-apoptotic protein (encoded by the *Bcl2* gene) that is present in a wide variety of hematolymphoid and nonhematolymphoid cells. Unlike adult human follicular lymphoma, the mouse follicular lymphoma is BCL-2 negative (Kunder et al., 2007). BCL-2 is not useful in diagnosing mouse lymphomas, as the protein is commonly expressed and often over-expressed in various mouse lymphoma types (Kunder et al., 2007).

CD10 (encoded by the *Mme* gene) is also known as common acute lymphoblastic lymphoma antigen (CALLA in humans). Hematolymphoid cells of humans express CD10, but those of mice do not. However, immunohistologic and flow cytometric analysis indicate that the distribution of CD10 in the nonhematolymphoid tissues of both species is identical (Kalled et al., 1995).

CD30 is an activation protein (encoded by the *Tnfrsf8* gene) that is a member of the tumor necrosis factor (TNF) receptor superfamily. It is expressed on human activated B cells, activated T cells, and pulmonary macrophages. However, in the mouse thymus, CD30 mRNA is highly expressed in cortical thymocytes, but the CD30 protein is expressed at only low levels (Schwarting et al., 1989, Bowen et al., 1996, Chiarle et al., 1999). Mouse CD30 is also expressed on activated T cells but on only relatively few medullary thymocytes (Chiarle et al., 1999). The CD30 staining pattern may be membranous, paranuclear, or both. Diffuse cytoplasmic immunoreactivity is considered an artifact unless there is also membrane or paranuclear staining. CD30 is reported to be expressed by Reed-Sternberg–like cells (atypical B cells) in mouse B-cell lymphomas with Hodgkin-like features (Raffegerst et al., 2009), similar to the CD30 expression seen in human Hodgkin lymphoma. We have recently confirmed membrane and paranuclear CD30 expression in normal activated B cells in germinal centers and that resting mouse B cells, T cells, or splenic macrophages do not express the protein. It now remains to be determined whether anti-CD30 antibody will be helpful in characterizing mouse lymphomas.

The CD40 antigen (encoded by the *Cd40* gene) is not lineage restricted. It is constitutively expressed on the surface of antigen presenting cells (APCs), including dendritic cells, macrophages, and mature and immature pro-B cells, but it is absent from early precursor pre-B cells and plasma cells.

CD117 (KIT, cKIT, encoded by the *Kit* gene), a tyrosine kinase receptor protein, is expressed on the surface of mouse pro-B blast cells and pro-T blast cells during normal lymphocyte maturation and in neoplastic lymphoblastic lymphoma/leukemia of the pro-B– and pro-T–type cells (Godfrey et al., 1992, Sykora et al., 1997, Escribano et al., 1998, Rehg et al., 2012). However, CD117 is not expressed on normal or neoplastic human pro-B blasts (Escribano et al., 1998).

CD127 (IL-7Ra, encoded by the *IL7R* gene) is expressed by immature B cells in the bone marrow and by double-negative (CD4−/CD8−) thymocytes, but not by double-positive (CD4+CD8+) thymocytes or mature peripheral T cells (Ceredig and Rolink, 2002, Mazzucchelli and Durum, 2007). This antigen marker is useful for characterizing the stage of B- and T-cell development and the immature stage of lymphoblastic lymphomas (Fig. 10.7).

CD138 (syndecan-1, encoded by the *Sdc1* gene) is a heparin sulfate–rich membrane glycoprotein that is expressed on lymphoid, mesenchymal, and epithelial cells. It is expressed on mouse B-precursor cells and is lost immediately before release of the immature B cells from the bone marrow into the circulation (Tung et al., 2006). CD138 is expressed in normal and neoplastic plasma cells and in mouse B-lymphoblastic lymphomas (O'Connell et al., 2004, Kunder et al., 2007, Rehg et al., 2012, Rehg et al., 2015). CD138 is reportedly expressed in the abnormal T cells in IgM-deficient MRL/lpr mice (Seagal et al., 2003). It is not expressed on normal T cells, myeloid cells, megakaryocytes, or erythroid cells. Immunohistochemical

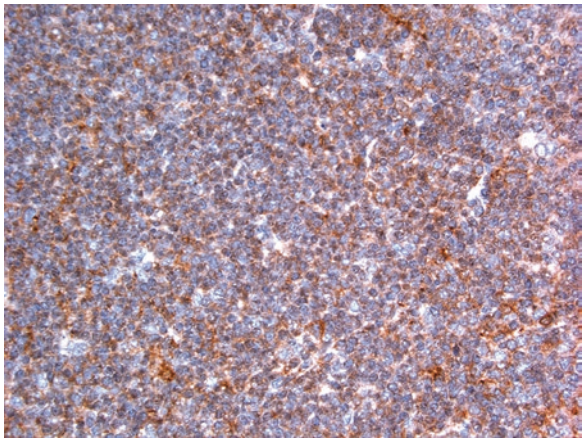

Fig. 10.7 A T-lympho blastic lymphoma of an adult B6,129 mouse expressing CD127 (IL7Rα) (Chromogen: DAB)

staining localizes CD138 to the cell membrane. However, the antigen may be undetectable if the tissue is not adequately fixed.

Peanut agglutinin (PNA) is a lectin on the surface of follicular germinal center cells, pre-B cells, and immature DP+ T cells of mice and other species (Rose and Malchiodi, 1981, Ree and Hsu, 1983, Newman and Boss, 1980, Rehg et al., 2012). Except for the thymus, the distribution of cells in the rat that express PNA is markedly different from that in the mouse (Rose and Malchiodi, 1981, Sharon, 1983).

Terminal deoxynucleotidyl transferase (TDT) transcripts (encoded by the *Dntt* gene) are not found in the cells of mice until 3–5 days after birth (Bogue et al., 1992). TDT in humans, bovines, rats, and mice is present in three isoforms (Thai and Kearney, 2004). Expression of all three of the human TDT isoforms is restricted to the nucleus. In contrast, only one of the mouse and rat TDT isoforms is expressed in the nucleus, and the other two isoforms are restricted to the cytoplasm (Di Primio et al., 1992, Bentolila et al., 1995, Thai and Kearney, 2004). In mouse and rat FFPE tissue, the nuclear expression is more intense than the cytoplasmic expression (Fig. 10.8). As detected by immunohistochemical staining a small percentage of the bone marrow population of immature B and T lymphocytes express TDT. It is expressed in the pro-B–cell stage of B-cell development and is downregulated in later stages (Chilosi and Pizzolo, 1995). TDT expression appears to be limited to the pro-B–lymphoblastic lymphomas in the mouse, whereas in humans the antigen is frequently expressed in all B- precursor cell stages (Rehg and Sundberg, 2008, Borowitz, 2008, Rehg et al., 2015). TDT is also expressed in all stages of T-cell development and in most T-lymphoblastic lymphomas in the mouse.

TDT expression may also be detected in human acute myeloid leukemia (AML), particularly in the MO subtype (Orazi et al., 1994). To date, no similar observation has been reported in rodents.

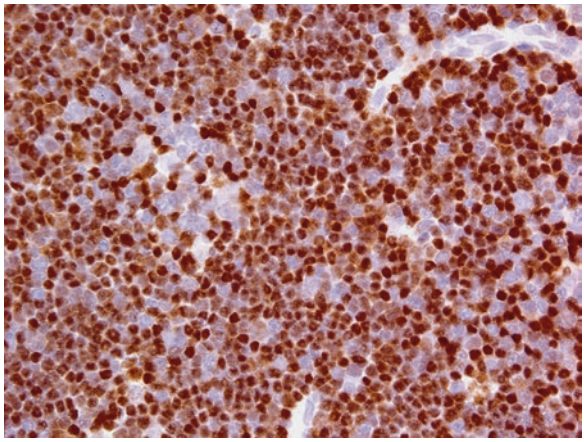

Fig. 10.8 Thymus of a young Sprague Dawley rat showing the cortical thymocytes with greater nuclear TDT expression than cytoplasmic TDT expression (Chromogen: DAB). Like those of rats, mouse cortical thymocytes express TDT in the nucleus and cytoplasm

10.7 Myeloid Lineage Antigens

The most useful myeloid markers depend on the differentiation and maturation stage of the myeloid cell population being studied (Rehg et al., 2012). The most common antibodies for detecting myeloid antigens in the mouse are listed in Table 10.2.

10.7.1 Undifferentiated Myeloid Antigens.

CD34 is a surface glycophosphoprotein (encoded by the *Cd34* gene) that is developmentally expressed in the early hematolymphoid stem cell stage. During normal development, a very small percentage of myeloid bone marrow cells, including granulocytic, monocytic, erythroid, and megakaryocytic precursors, are CD34 positive. However, anti-CD34 antibody labeling of mouse myeloid precursors appears to be CD34 clone specific (authors' experience). In mice, CD34 is also present on progenitor and mature mast cells and on basophils (Arinobu et al., 2005). When CD34 is used as a marker to harvest mouse bone marrow stem cells, the stem cell culture may revert to a pure culture of mast cells upon being grown in subculture (author's experience). Therefore, CD34 should not be used as a marker to identify mouse stem cells for bone marrow transplantation. CD34 antigen is also expressed on some blood vessel endothelial cells, but not on large veins, large arteries, or the lining cells of the vascular sinuses of the spleen and placenta. With immunohistochemical staining, CD34 antigen shows a cell membrane expression pattern and is an excellent marker of undifferentiated myeloid leukemia (Rehg et al., 2012). Unlike most other CD antigens, CD34 is expressed on many malignant and benign nonhematopoietic tumors (Chu and Weiss, 2009).

CD43, also known as leukosialin and sialophorin (encoded by the *Spn* gene), is a protein that is present on hematopoietic progenitors (Rehg et al., 2012). The antigen is expressed in acute myeloid leukemia, most T lymphomas/leukemias, pro-B–lymphoblastic lymphoma/leukemia, and plasma cell diseases of mice (Rehg et al., 2012, Rehg et al., 2015).

CD117 (encoded by the *Kit* gene) is a tyrosine kinase receptor protein that is expressed on the surface of bone marrow precursors (myeloid and erythroid precursors, pro-T blasts, and mouse pro-B blasts, but not human pro-B blasts) and immature and mature mast cells (Sykora et al., 1997, Arber et al., 1998, Chu and Weiss, 2009, Escribano et al., 1998). In the mouse, CD117 is expressed on the cells of pro-B– and pro-T–cell lymphoblastic lymphomas/leukemias and myeloid leukemias of the undifferentiated type (Godfrey et al., 1992, Rehg et al., 2012). It is also a useful marker in diagnosing mast cell disorders and gastrointestinal stromal tumors (Arber et al., 1998).

10.7.2 Common Granulocyte and Monocyte Antigens

Myeloperoxidase (MPO, encoded by the *Mpo* gene) is a constituent of neutrophil primary granules. It is expressed in the immature neutrophil stages and is downregulated as they mature (Pinkus and Pinkus, 1991, Zhu, 1999). It is also expressed in the immature and mature stages of eosinophils (Pinkus and Pinkus, 1991, Zhu, 1999). Mouse and rat neutrophils have only one-fifth as much MPO as is present in human neutrophils (Rausch and Moore, 1975). The staining pattern is cytoplasmic and often granular. Erythroid precursors, megakaryocytes, mast cells, lymphoid cells, and plasma cells are not immunoreactive for MPO. Cells of the monocytic lineage react variably (typically weakly positive or nonreactive), but MPO is not expressed in resident macrophages (Tacchini-Cottier et al., 2000). MPO is a sensitive and specific marker of the myeloid lineage in leukemias. Mouse and human Kupffer cells, as well as human activated microglia, have also been reported to express MPO (Nagra et al., 1997, Brown et al., 2001, Rensen et al., 2009). However, the authors have not observed MPO expression by these cells in rodents, and rat Kupffer cells have been reported by others to not express MPO(Amanzada et al., 2011).

CD68 is a glycoprotein (encoded by the *Cd68* gene) that is associated with lysosomes and is expressed in the cytoplasm of most cells of the monocyte/macrophage lineage (Rehg et al., 2012). Intracytoplasmic immunoreactivity with anti-CD68 antibody is observed in Schwann cells of nerves with Wallerian degeneration and in Schwann cells of traumatic neuromas, neurofibromas, and granular cell tumors (Kaiserling et al., 1993). Anti-CD68 antibody also reacts with melanocytes, dendritic cell subsets, mast cells, and NK cells (Weiss et al., 1994, Li et al., 1996). The monoclonal anti-CD68 antibodies KP1 and PG-M1 react with monocytes/macrophages. Whereas KP1 reacts with human monocytes/macrophages and myeloid progenitor cells, PG-M1 reactivity is restricted to the monocyte/macrophage lineage (Falini et al., 1993). Our experience suggests that the rat anti-mouse CD68

(clone FA-11) antibody and the rat anti-CD68 (clone ED1) antibody react with monocytes/macrophages, but not with other myeloid cells. These clonal differences in the expression pattern associated with neutrophils strongly suggest that the specificity of an anti-CD68 antibody should be assessed before use.

The Ly6 haplotype family, which includes the Ly6B and Ly6G haplotypes, has many members. Mouse strains 129, AKR, C57BL/6, C57BL/10, C58, DBA, and SJL express the LY6B haplotype antigen, whereas mouse strains A2G, A/Sn, ASW, BALB/c, C3H/He, and CBA express the LY6G haplotype antigen (Hirsch and Gordon, 1983, Rosas et al., 2010).

The LY6B haplotype (encoded by the *Ly6b* gene) is synonymous with the 7/4 antigen, a differentiation antigen associated with neutrophils and monocytes that is lost as monocytes differentiate into macrophages and is not expressed by mature mouse tissue-resident macrophages. Immature and mature neutrophils and monocytes label with monoclonal antibody 7/4 , but the monoclonal antibody does not label macrophages, eosinophils, mast cells, erythroid cells, or lymphocytes (Hirsch and Gordon, 1983).

In mice, the LY6G haplotype (encoded by the *Ly6g* gene) is expressed by neutrophils, a subset of eosinophils, and dendritic cell subsets and is transiently expressed during the developmental stages of monocytes (Lagasse and Weissman, 1996, Lopez et al., 1984). The monoclonal anti-LY6G antibody NIMP-R14 labels neutrophils, developing monocytes, and eosinophil subsets, but the antibody does not label macrophages, mast cells, erythroid cells, or lymphocytes (Nagendra and Schlueter, 2004).

Lysozyme (encoded by the *Lyz1* gene) is a marker of myeloid cells, monocytes/histiocytes, and their neoplasms (Hard and Snowden, 1991, Ward and Sheldon, 1993). Its immunohistochemical staining pattern is cytoplasmic and often granular.

10.7.3 Erythroid Cell Antigens

GATA1 (encoded by the *Gata1* gene) is a transcription factor expressed in the nuclei of erythroid cells, megakaryocytes, eosinophils, mast cells, and basophils (Zon et al., 1991, Rehg et al., 2012). GATA1 is reportedly not expressed in immature mouse neoplastic mast cells (Zon et al., 1991), but it is expressed in erythroid and megakaryocytic leukemia cells (Hao et al., 2006, Torchia et al., 2007, Rehg et al., 2012). The authors have found GATA1 expression to be inconsistent in mast cells in FFPE tissues.

Glycophorin A (CD235a, encoded by the *Gypa* gene) is a major sialoglycoprotein of the erythrocyte membrane. In mice and rats, the intensity of glycophorin A expression increases as cells mature, and it accumulates during the induction of murine erythroleukemia (Matsui et al., 1985, Kina et al., 2000). Anti-glycophorin A antibody does not label myeloid cells, lymphocytes, or their respective neoplasms. Erythroid developmental stages from late erythroblasts to mature erythrocytes strongly express glycophorin A (Rehg et al., 2012), whereas it is only weakly expressed in early erythroblasts (Sadahira et al., 1999).

TER119 (encoded by the *Ly76* gene) is a membrane molecule associated with cell surface glycophorin A, but it is not identical to glycophorin A. Cell surface membrane expression occurs in the proerythroblasts and extends to the mature erythrocyte stage (Kina et al., 2000). Other myeloid progenitor cells, mast cells, and lymphocytes do not express TER119 protein; neither do murine erythroleukemia cell lines nor erythroid leukemia cells (Kina et al., 2000, Torchia et al., 2007, Rehg et al., 2012).

10.7.4 Megakaryocyte Antigens

Megakaryocytes are reported to express a variety of antigens, including CD34, CD41, CD61, CD79a, clusterin, GATA1, RUNX1, and von Willebrand factor (McComb et al., 1982, Debili et al., 1992, Elagib et al., 2003, Rehg et al., 2012). Although human megakaryocytes are reported to express CD31 (Muler et al., 2002, Pusztaszeri et al., 2006), the authors have not observed CD31 expression in mouse megakaryocytes. CD34, CD41, CD61, GATA1, RUNX1, and von Willebrand factor (vWF) are the most useful markers for identifying normal and abnormal megakaryocytes (Chuang et al., 2000, Hao et al., 2006, Rehg et al., 2012). However, CD34 and CD79a labeling of megakaryocytes is clone specific. Unlike the anti-CD34 clone Ram34 antibody, the clone MEC14.7 antibody does not label megakaryocytes (Rehg et al., 2012). CD79a is not expressed in normal myeloid cells, but anti-CD79a (clone HM57) antibody has been reported to label normal human megakaryocytes (Chuang et al., 2000, Bhargava et al., 2007). The authors have observed that the anti-CD79a (clone HM57) antibody labels mouse megakaryocytes (Rehg et al., 2012); however, the authors and others have also observed that the anti-CD79a (clone JCB117) antibody does not label megakaryocytes (Bhargava et al., 2007, Rehg et al., 2012). The detection of CD34 and CD79a expression in megakaryocytes appears to be antibody clone dependent; this probably results from different epitopes being detected by the specific antibody clones. Alternatively, the antibodies to CD34 and CD79a may be cross-reacting with antigens of an undetermined nature.

CD41 (encoded by the *Itga2b* gene) expression pattern is cytoplasmic early in megakaryocyte maturation (Gewirtz, 1995). Antibodies to the CD41 and CD61 antigens are very helpful in immunophenotyping mouse megakaryocytes, platelets, and murine megakaryocyte leukemia (Hisa et al., 2004, Hao et al., 2006).

CD61 (encoded by the *Itgb3* gene) has an essential role in platelet aggregation as a receptor for von Willebrand factor. Most megakaryocytic leukemias, as well as normal platelets and megakaryocytes, express CD61 (Hao et al., 2006).

RUNX1, also known as AML1, is a transcription factor that regulates the differentiation of several hematopoietic lineages. The RUNX1 protein (encoded by the *Runx1* gene) has a nuclear expression pattern in lymphoid and myeloid cells, but is not expressed in adult erythroid cells (Corsetti and Calabi, 1997, Elagib et al., 2003). Anti-RUNX1antibody can aid in differentiating between megakaryocytic

and erythroid leukemia if it is included in a panel of antibodies that label the erythroid and megakaryocyte antigens discussed in this section (Hao et al., 2006, Rehg et al., 2012).

Von Willebrand factor (vWF, encoded by the *Vwf* gene), also known as Factor VIII-related antigen, is a common endothelial cell marker. vWF, CD41, and CD61 are sensitive and specific markers for megakaryocytes and platelets (Chuang et al., 2000, Rehg et al., 2012), and together these three markers are useful in diagnosing megakaryocyte disorders and leukemia. However, it should be noted that vWF may not be expressed in the early megakaryoblasts of some leukemias.

10.7.5 Eosinophil Antigens

Under some circumstances, light microscopy with hematoxylin and eosin staining cannot reveal whether a granulocyte is a neutrophil or an eosinophil (Rehg et al., 2012). Myeloperoxidase is not helpful in differentiating between a neutrophil and an eosinophil, as MPO is expressed in both of these granulocytes (Rehg et al., 2012). The histochemical stains Astro blue/vital new red, Congo red, Sirius red and Luna are reported to stain eosinophils. We have used the Luna stain to determine whether a cell is an eosinophil, with variable results (Rehg et al., 2012). It appears that consistent quality with the Luna stain depends on the differentiation step of the Luna procedure. Therefore, immunohistochemical staining is the preferred approach. Immunohistochemical staining for eosinophil peroxidase requires frozen tissue. However, antibody to eosinophil granule major basic protein (encoded by the *Prg2* gene) is very effective at labeling eosinophils in FFPE tissue (Rehg et al., 2012).

10.7.6 Mast Cell and Basophil Antigens

Mast cell precursors migrate from the bone marrow to peripheral tissues as morphologically unidentifiable agranular mast cells. After arriving in these peripheral tissues, they undergo terminal differentiation into identifiable mast cells (Hallgren and Gurish, 2007). Two mast-cell subtypes have been distinguished in rodents on the basis of differences in their fixatives, the proteases they express, and their histochemical staining properties (Welle, 1997). Cells of the first subtype, the atypical, T cell-dependent mucosal mast cells (MMCs), are reported to be found mainly in the mucosa of the gastrointestinal tract and the respiratory tract. There are two intestinal MMCs in the mouse, an interepithelial MMC (IEMMC) and a lamina propria MMC (LPMMC, JER, unpublished observation). The IEMMC has prominent eosinophilic globular granules and the LPMMC is not readily apparent in FFPE H&E stained tissue. The proliferation of T cell-dependent MMCs is mediated by the cytokines IL-3, −4, −9, and −10. Cells of the second subtype, the T cell-independent connective tissue mast cells (CTMCs), reside mainly in the skin, the submucosa, muscularis propria, and

serosa of the gastrointestinal tract, and the peritoneum. Fibroblast-derived factors, e.g., stem cell factor (SCF), appear to mediate the development of this subtype.

In addition to their respective T cell-dependent and -independent features, the two mast-cell subtypes are distinguished by their histamine content, proteoglycan, and size. The MMC granules are relatively soluble and contain chondroitin sulfate and little histamine. Their anionic site is blocked by aldehyde; therefore, special fixation and staining conditions are required to reveal the LPMMCs. MMC granules often become resistant to metachromatic staining after fixation with some formalin-based fixatives (Enerback, 1981). Rapid penetrating fixatives and strongly acid staining conditions are ideal for demonstrating MMC metachromasia. The heparin and histamine content of the CTMC granules allows for metachromatic staining of this class of mast cells with basic dyes, such as toluidine blue or Giemsa.

CD34 and CD117 (KIT, c-KIT) are membrane antigens, and GATA1 is a nuclear antigen (Rehg et al., 2012). Consequently, the staining features of these antigens are not dependent on the biologic characteristics of the mast cell granules, nor is the immunoreactivity of histamine and tryptase affected by formalin-based fixatives (Kunder et al., 2007). Therefore, antibodies to CD34, CD117, and GATA1 can be used to identify both MMC and CTMC precursors and degranulated mast cells when standard histochemical approaches that use toluidine blue, Giemsa, or Leder (chloroacetate esterase) stains are ineffective (Drew et al., 2002). MMCs and CTMCs are equally dependent on SCF and CD117 for their development and maturation, and both express CD34, which further supports the use of CD117 and CD34 as excellent pan-mast cell markers for immunohistochemical analysis (Drew et al., 2002, Hallgren and Gurish, 2007).

CD68, is a common macrophage marker and it is also expressed in mast cells, but it is considered a non-specific marker for them (Weiss et al., 1994, Li et al., 1996).

Histamine is an organic nitrogen protein that is produced in the cytosol and stored in the cytoplasmic granules of mast cells and basophils in mice, rats, and other species. Histamine elicits an inflammatory response after IgE triggers its release from sensitized mast cells and basophils. Immunohistochemical staining with antihistamine antibody is used to detect mast cells in tissues (Rehg et al., 2012). Histamine that is not associated with mast cells is found in several tissues, such as the brain and the gastric enterochromaffin-like cells.

Mast cell protease 1 (MCP-1, encoded by the *Prss34* gene) is a β-chymase that is stored and secreted in a tissue-specific manner by MMCs. It is the only chymase expressed by intestinal IEMMCs and it is not expressed by the LPMMCs and the CTMCs. Mast cell protease 11 (MCP-11) is its rat counterpart. Unlike mouse IEMMCs, which preferentially express MCP-1, mouse basophils preferentially express MCP-11 (Ugajin et al., 2009). MCP-1 exhibits a cytoplasmic expression pattern with immunohistochemical staining (Fig. 10.9).

Tryptase, a cytoplasmic serine protease (encoded by the *Prss32* or *Prss33* genes) is expressed almost exclusively in mast cells. Formalin-based fixatives do not affect tryptase immunoreactivity in FFPE tissue (Rehg et al., 2012). Tryptase is reported to be a helpful marker in diagnosing mast-cell neoplasms (Kunder et al., 2007); however, the value of tryptase as a marker for diagnosing LPMMC neoplasms in

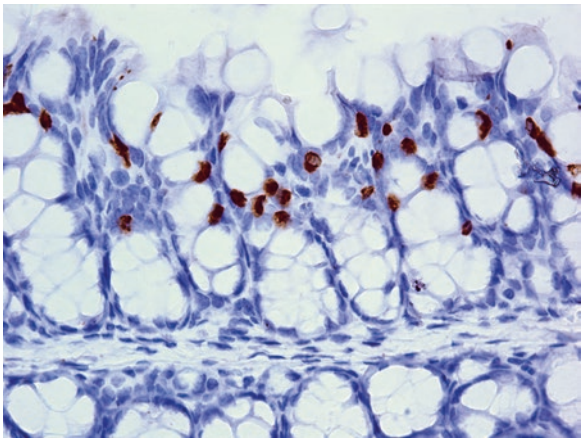

Fig. 10.9 Colon of a Foxp3-knockout adult mouse showing MCP-1 expression in interepithelial mucosal mast cells (Chromogen: DAB)

FFPE tissue remains to be determined, because only a few MMCs are reported to express tryptase (Chen et al., 1993). We have observed that labeling of tryptase is highly sensitive and specific for LPMMCs and CTMCs, but that IEMCs do not label for tryptase. Both intestinal MMCs (IEMMC, LPMMC) and CTMCs express CD34 and CD117 (JER, unpublished observation). Therefore, CD34 and CD117 are considerated the preferred pan–mast cell markers in immunohistochemical analysis. All anti-CD117 antibodies are not equal in their ability to label the cell lineages that express the antigen. Therefore, it is important to verify that the anti-CD117 antibody of choice labels the cell(s) of interest (JER, unpublished observations).

Mast cells and basophils are functionally and developmentally similar cell types. Circulating mouse basophils are present at very low levels in the peripheral blood and are not normally found in mouse tissues. However, both mast cells and basophils are reported to be associated with T-helper type 2 responses involving the skin, lungs, and gastrointestinal tract and with other autoimmune inflammatory disorders (Obata et al., 2007, Nabe et al., 2013, Voehringer, 2013). It is difficult to distinguish mast cells from basophils in tissues based on morphological criteria. Both cells contain dark basophilic granules, which can impede an adequate determination as to whether a cell is a monolobed mast cell or a bilobed basophil. The metachromatic granules of both cell types are water soluble, and they can be diminished or totally abolished by conventional tissue processing, especially with an acidified decalcification solution. Furthermore, in pathologic conditions of humans, the morphology of the two cell types may be altered; basophils may have a mononuclear morphology, and mast cells can have a bilobed or multilobed nucleus (Horny et al., 2011). Mouse mast cells can also exhibit varied nuclear profiles, having both monolobed nuclei and polylobed nuclei (Gurish et al., 1997). Therefore, immunohistochemical and histochemical analysis may be the only means of determining whether a cell or a proliferation of cells is of mast cell or basophil lineage. Human basophils and mast cells do not express CD34 (Horny et al., 2011); however, immature and mature mouse mast cells do express CD34, as do basophil progenitors (Arinobu et al.,

2005). There is currently no evidence as to whether immature or mature mouse basophils express CD34. Generally, basophils, unlike mast cells, do not express tryptase (Agis et al., 1996). However, in pathologic conditions, human basophils may show positive immunohistochemical staining for tryptase (Li, 2007). Unlike mast cells, mouse basophils are the major source of mouse mast-cell tryptase 11 (mMCP11) (Ugajin et al., 2009, Yamagishi et al., 2011). Because of the difficulties discussed above, flow cytometry has been commonly used to identify the mast cells and basophils associated with Th2-type hypersensitivity in mice. It has been reported that the basophils in several flow cytometry studies appeared to lack granules, which is inconsistent with these cells being normal mouse basophils (Lee and McGarry, 2007). There are several possible reasons for this discrepancy. For example, it is possible that, as reported in humans, the morphology of mouse basophils may be altered in pathologic conditions. To the authors' knowledge, there has been no definitive study in mice that correlated the morphologic changes of mast cells or basophils in disease states, as seen in FFPE specimens, with a concurrent flow cytometry analysis in the same study mice. Therefore, based on the literature, we believe that CEA, CD68, CD117, and mMCP11 will be the most helpful markers for differentiating between mast cells and basophils in FFPE mouse tissues.

10.7.7 Tissue Histiocyte (Macrophage) Antigens

Macrophages display a wide variety of phenotypes, depending on the cytokine environment, the tissue localization (Rehg et al., 2012), and the time point in the inflammatory process. Consequently, there are multiple heterogeneous macrophage subsets, necessitating the use of a panel of antibodies to confirm their macrophage lineage (Gordon et al., 2014). The more common tissue macrophage biomarkers are CD68, CD163, F4/80, IBA1, MAC2, and MAC3 (Table 10.3). Other antigens expressed by macrophages are CD45, lysozyme, and (rarely) S100P.

CD68 is an intracellular glycoprotein (encoded by the *Cd68* gene) that is present in lysosomes and phagosomes and is expressed in the cytoplasm of most tissue macrophages. Although the antigen is used as a macrophage marker, it is not a specific marker for cells of the monocyte/macrophage derivation, as other cells that contain lysosomes or phagosomes, such as mast cells or melanocytes, may be immunoreactive (Weiss et al., 1994).

CD163 is a glycoprotein (encoded by the *Cd163* gene) that belongs to the scavenger receptor cysteine-rich superfamily (Lau et al., 2004). Its expression is largely restricted to monocytes and tissue macrophages. CD163 immunoreactivity is observed in resident macrophages of all normal tissues, including Kupffer cells, except for splenic white pulp and germinal center tingible body macrophages. Labeling with anti-CD163 antibody is a more specific marker of disorders of monocyte/macrophage origin than is labeling with anti-CD68 antibody. In the brain, CD163 expression, unlike that of IBA1, is restricted to the perivascular, meningeal, and choroid plexus macrophages (Fig. 10.10) (Kim et al., 2006, Perry et al., 1994).

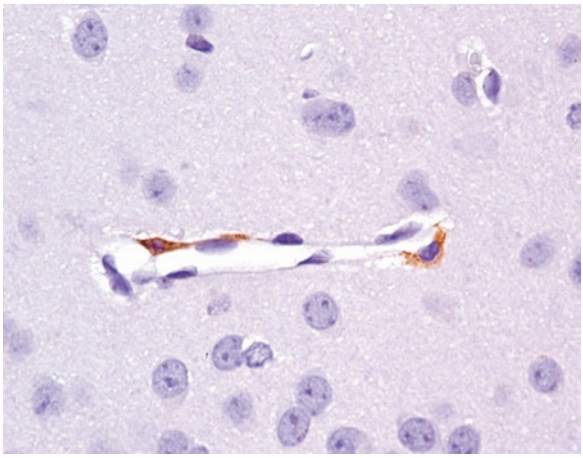

Fig. 10.10 Brain of an adult FVB mouse showing two perivascular macrophages with cytoplasmic expression of CD163 (Chromogen: DAB)

F4/80 is a glycoprotein (encoded by the *Adgre1* gene) that has 80% surface membrane expression and 20% cytoplasmic expression (Gordon et al., 1992, Perry et al., 1992). F4/80-expressing macrophages in FFPE lymphoid organs are restricted to splenic red pulp, lymph node medullary cords, and the thymic corticomedullary junction (Rehg et al., 2012). Liver Kupffer cells and alveolar macrophages express F4/80, but the level of expression in alveolar macrophages is low (Malorny et al., 1986). Circulating monocytes express very low levels of F4/80, and it is often not detectable by immunohistochemical staining; neutrophils and lymphocytes also do not express the antigen (Malorny et al., 1986, Taylor et al., 2005, Francke et al., 2011). Caution is warranted when evaluating inflammatory tissues, because interferon gamma (IFNγ) down-regulates F4/80 expression. Consequently, macrophages may not be labeled for F4/80 in inflammatory lesions with IFNγ-secreting lymphocytes and/or NK cells. Anti-F4/80 antibody is also reported to label eosinophils and neovascularized tissue endothelial cells (McGarry and Stewart, 1991, Gordon et al., 1986, Gouon-Evans et al., 2002). These factors need to be considered when assessing cellular infiltrates in tumors or inflammation.

Ionized calcium–binding adapter molecule 1 (IBA1, encoded by the *Iba1* gene) is the same antigen as the allograft inflammatory factor 1 (AIF-1) antigen. Immunohistochemical staining shows the IBA1 protein to have a nuclear and cytoplasm pattern of expression. Although IBA1 was initially recognized as a microglial marker, it is now considered a pan-macrophage marker. It is expressed on monocytes (Imai et al., 1996, Jeong et al., 2013) and all subpopulations of tissue macrophages examined to date except alveolar macrophages (Kohler, 2007). In FFPE tissue, IBA1 is an excellent marker of spleen marginal zone macrophages and marginal zone sinus metallophilic macrophages (Rehg et al., 2012).

Lysozyme is expressed in the cytoplasm of mouse and rat hematopoietic and non-hematopoietic cells (Speece, 1964, Klockars and Osserman, 1974). The level and intensity of lysozyme expression progressively increases as granulocytes differentiate from the late promyelocyte stage to the more mature granulocyte stages.

The antigen is a useful but non-specific marker of granulocytes, monocytes, tissue macrophages, and their neoplasms (Ward and Rehm, 1990, Ward and Sheldon, 1993, Pileri et al., 2002, Hao et al., 2010). Lysozyme expression is stronger in the alveolar macrophages than in other tissue macrophages, and its expression in tissue histiocytic sarcomas is variable (Klockars and Osserman, 1974, Ward and Sheldon, 1993). Therefore, to avoid false negatives with immunohistochemical staining, liver should be used instead of lung when developing a tissue lysozyme assay.

The renal proximal convoluted tubules are the most noted nonhematopoietic cells to contain lysozyme. Immunohistochemical staining sometimes reveals large volumes of the enzyme in the renal tubules in association with certain rodent histiocytic sarcomas, histiocyte-associated B-cell lymphomas, and myeloid leukemias (Klockars et al., 1974, Hard and Snowden, 1991, Rehg et al., 2015). This finding is likely to be associated with renal tubular absorption of glomerular filtration fluid containing lysozyme that has been released into the blood by the tumor cells or tumor associated histiocytes.

MAC2 (galectin 3, encoded by the *Lgals3* gene) is the intracellular protein galactose-specific lectin 3. MAC2 protein expression correlates with the maturation of a macrophage subset (Leenen et al., 1994). Upon activation, MAC2 is an inducible component of the macrophage cell surface. Depending on the cell type, MAC2 can exhibit a membranous, cytoplasmic, or nuclear distribution pattern by immunohistochemical staining (Frigeri and Liu, 1992, Hao et al., 2010). MAC2 is not expressed on lymphoid cells, but it is expressed on all resident tissue macrophages, as well as on splenic marginal zone macrophages, splenic marginal zone sinus metallophilic macrophages, follicular dendritic cells (FDCs), and thymic macrophages (Table 10.7) (Rehg et al., 2012). MAC2/e binding protein expressed on neutrophils will immunoreact with some anti-MAC2 antibodies. The antigen expression is also associated with the epithelium of the bronchi, renal tubules, intestine, skin, hair follicles, choroid plexus, and ependyma.

MAC3 is a glycoprotein (encoded by the *Lamp2* gene) that is found on macrophages, dendritic cells, megakaryocytes, and granulocytes, but it is not expressed on any other type of hematolymphoid cell. Epithelial and endothelial cells in a variety of tissues also express the glycoprotein (Flotte et al., 1983). MAC3 expression in lymphatic and vascular endothelial cells is problematic when one is interpreting the immunoreactivity pattern of MAC3 in lymphoid organs. Both Kupffer cells and liver hepatocytes are labeled by anti-MAC3 antibodies.

The S100 protein (S100P, encoded by the *S100b* or *S100a1* gene) is dimeric, comprising alpha and beta subunits. Consequently, the protein consists of three isoforms: S100ao (alpha dimer), S100a (alpha-beta isoforms) and S100b (beta dimer). The S100P preparation that is generally used to generate anti-S100P antibody has much more beta subunit than alpha subunit. Therefore, the localization of S100P by immunohistochemical staining is actually an indicator of the beta subunit (containing S100a [αβ] and S100b [ββ]) but not of the S100ao protein (Takahashi et al., 1984). S100P is expressed in Langerhans cells, interdigitating dendritic cells (IDCs), and their respective neoplasms. Ordinary macrophages, alveolar macrophages, FDCs, and blood monocytes contain only the alpha subunit. Consequently, most anti-S100P antibodies label these cells very weakly or not at all. Depending on the antibody used, FDCs may also be labeled with anti-S100P antibody (Chilosi et al., 1985, Carbone et al., 1986). Thirty-three percent of

Table 10.7 Immunophenotype markers of mouse dendritic cells and macrophages[a]

Markers	Conventional dendritic cells	Follicular dendritic cells	Interdigitating dendritic cells	Langerhans cells	Plasmacytoid dendritic cells	Macrophages	Monocytes
CD4	+/−[b]	−	+	+	+/−	−	−
CD8	+/−	−	+	+/−	+/−	−	−
CD11b	+/−	−	−	+	−	+/−	+
CD11c[c]	+	−	+	+	+/−	+/−	+
CD21/35	−	++	−	−	−	−	−
CD40	+	+	+	+	+	+	+
CD45	+	−	+	+	+	+	+
CD45R/B220	−	−	−	−	+	−	−
CD163	−	−	−	−	−	++	+
CD68	+/−	+/−	+	+	+/−	+	+
F4/80	−	−	−	+/−	UR[d]	+/−	+/−
Fascin	+	+++	++	−	−	+/−	UR
IBA1	++	−	−	+/−	UR	++	+
Langerin	+/−	−	−	++	−	−	−
Lysozyme	−	−	−	+/−	−	+	+/−
MAC2	+	+/−	+	+	UR	+/−	+
MHCII	+	−	++	+	+/−	+	−
S100	−	+/−	++	++	−	+/−	−

[a]Sources: (Hume et al., 1983, Leenen et al., 1998, Shortman and Liu, 2002, Asselin-Paturel et al., 2003, McKenna et al., 2005, Kohler, 2007, Orsmark et al., 2007, Hume, 2008, Haniffa et al., 2013, Gordon et al., 2014, Schlitzer and Ginhoux, 2014)

[b]Expression: − None, + present, ++ high, +/− low or negative depending on subset or cell activation

[c]Requires frozen tissues or flow cytometry; all other markers can be detected on FFPE tissue

[d]Unreported

human histiocytic sarcomas express S100P (Pileri et al., 2002), but we have rarely observed its expression in mouse histiocytic sarcoma (Rehg et al., 2012). The incidence of S100P expression in rodent histiocytic sarcomas warrants further investigation. The level of S100P in rodent tissues is less than that in humans (Pedrocchi et al., 1993, Davies et al., 1995), which may account for the low incidence of S100P expression in rodent histiocytic sarcomas. There are contradictions in the literature and the commercial data sheets regarding the acceptable distribution pattern for positive S100P immunoreactivity. Some data sheets specify cytoplasmic expression to be the correct expression pattern, whereas others specify nuclear staining with or without cytoplasmic staining to be correct (Chu et al., 1999), and others report that S100P immunoreactivity should be both nuclear and cytoplasmic in order to be valid (Wick, 2006).

Arginase 1 (encoded by the *Arg1* gene) is a cytosolic enzyme that has a cytoplasmic and patchy nuclear expression pattern with immunohistochemical staining (Rehg et al., 2012). The enzyme catalyzes the conversion of arginine to ornithine. The protein is a biomarker of M2 macrophages activated by the CD4 Th2 cytokines IL-4 and IL-13, which are associated with the alternative macrophage activation pathway.

Inducible NO synthase 2 (iNOS2) is a cytoplasmic enzyme expressed at very low or undetectable levels in resting rodent neutrophils and macrophages. The expression of iNOS2 is greatly upregulated in neutrophils and macrophages upon their activation by the CD4 Th1 pro-inflammatory cytokines TNFα and IFNγ (McCafferty et al., 1999, Richter et al., 1999, Nair et al., 2003, Rehg et al., 2012). Macrophages expressing iNOS2 that are activated via the classical CD4 Th1 pathway are known as M1 macrophages.

YM1 (CHI3L3, encoded by the *Chil3* gene) is a member of the chitinase family of proteins, but it does not have chitinase activity. The YM1 protein is expressed in normal neutrophils and macrophages activated by the cytokines IL-4 and IL-13,and it is associated with the CD4 Th2, IL-4, IL-13 alternative macrophage pathway (Ward et al., 2001, Hung et al., 2002, Nio et al., 2004, Milner et al., 2010, Rehg et al., 2012). Macrophages activated by the CD4 Th2 alternative pathway are known as M2 macrophages. Under normal conditions, lung alveolar macrophages constitutively express YM1 and activated M2 macrophages transiently express YM1 (Ward et al., 2001, Hung et al., 2002, Milner et al., 2010, Rehg et al., 2012). Like lysozyme, the YM1 protein has been reported to accumulate in the cytoplasm of renal tubules in a mouse with myelogenous leukemia (Marchesi et al., 2006). In H&E-stained lung sections, eosinophilic YM1 protein crystals can generally be differentiated from hemoglobin crystals based on their color, location, and shape. Differentiating between these two crystals can be more problematic in rats than in mice. Hemoglobin crystals are typically rectangular in shape, are generally located in the extracellular space, and are the color of red blood cells, whereas YM1 protein crystals may have tapered spindle and/or rectangular shapes, are located within cells and in the extracellular space, and are typically deeply eosinophilic. On occasion, it may be difficult to determine whether pulmonary crystals are of the hemoglobin or YM1 protein type. In these instances, immunohistochemical staining can be helpful: YM1 protein crystals will be labeled with YM1 and hemoglobin crystals will not.

10.7.8 Dendritic Lineage Antigens

Dendritic cells are of bone marrow and mesenchymal cell origin. Most are of bone marrow origin, but FDCs are believed to be of mesenchymal origin. Dendritic cells of bone marrow origin are antigen-presenting cells (APCs). As such, they present processed antigen peptides that are bound to MHC molecules to naïve T cells, resulting in T-cell activation. However, FDCs present unprocessed antigen in the form of an immune complex to follicular B cells, and upon activation the B cells migrate to the T cell/B cell border of the follicle. Here, they present antigen to T-helper cells and receive co-stimulation, undergo proliferation, and form germinal centers in conjunction with FDCs, leading to plasma cell and memory B cell populations. The dendritic cells are a heterogeneous population, and though the various subtypes share many of the same antigens, they differ in their expression of some antigens that are more specific for particular subtypes, as depicted in Table 10.7. A panel of antibodies to CD8, CD21/35, CD45/B220, CD163, langerin, lysozyme, and S100 will greatly assist in differentiating the subsets of dendritic cells.

CD11b protein (encoded by the *Itgam* gene) is constitutively expressed by monocytes, tissue macrophages, dendritic cell subsets, granulocytes, NK cells, and memory B cells. It is not expressed on FDCs, IDCs, or Langerhans cells. CD11b is often used in flow cytometry to identify these different cell populations. However, because of the broad spectrum of cells that express this antigen and the fact that many of these cells are located in the same tissue, we do not recommend using anti-CD11b antibody for routine immunohistochemical analysis of tissue.

CD11c protein (encoded by the *Itgax* gene) is constitutively expressed by monocytes, tissue macrophages, dendritic cell subsets, granulocytes, and Kupffer cells. T and B cells and NK cells also express CD11c, but neither cutaneous Langerhans cells nor FDCs express the antigen. Flow cytometry is commonly used to identify dendritic cells, and detection of the protein by immunohistochemical staining requires frozen tissue.

The CD21/35 complex (encoded by the *Cr2* gene) in the mouse marks receptors for the C3b and C3d complement components. This complex is most strongly expressed in the cytoplasm of germinal center FDCs (Rehg et al., 2012), their neoplasms, and a subset of B cells. B precursors, immature B cells, resting and activated T-cells, monocytes/macrophages, granulocytes, and NK cells do not express the antigen complex. Anti-CD21/CD35 complex antibody is helpful for demonstrating the association of germinal centers in follicular lymphoma and the presence and character of germinal centers within lymphoid proliferations. In humans, CD21 is a marker of splenic littoral cell angioma, a tumor of the cells lining the splenic sinusoids (Arber et al., 1997, Neuhauser et al., 2000). It remains to be determined whether CD21 is a marker for the same tumor in rodents.

Fascin 1 (encoded by the *Fscn1* gene) is an actin-bundling protein expressed in the cytoplasm of FDCs, IDCs, and lymph node subcapsular sinus dendritic cells. Fascin 1 is also expressed variably in vascular endothelial cells and in the epithelium of a variety of organs (Zhang et al., 2008). It is not expressed in lymphocytes,

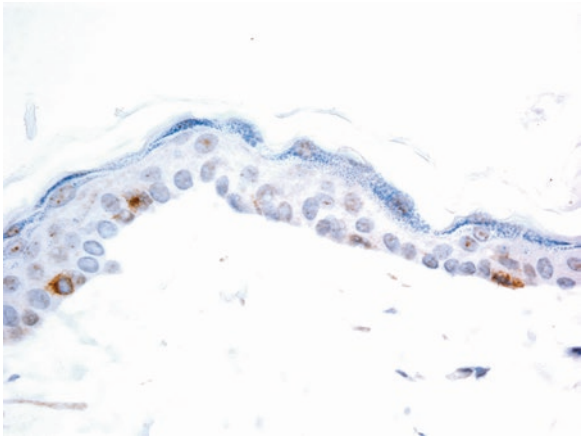

Fig. 10.11 Skin of an adult mouse showing three epidermal Langerhans cells in the basal cell layer with short dendritic and cytoplasmic expression of langerin (Chromogen: DAB)

myeloid cells, plasma cells, or normal Langerhans cells, but it is expressed by cells of human Langerhans cell histiocytosis (Pinkus et al., 2002). FDC sarcoma and IDC sarcoma also express fascin 1.

Langerin (CD207, encoded by the *Cd207* gene) is a transmembrane C-type lectin that is expressed in the cytoplasm and on the cell membrane of epidermal Langerhans cells (Fig. 10.11), migrating Langerhans cells, thymic medullary dendritic cells, and subsets of dendritic cells that are located in the peripheral lymph nodes, spleen, marginal zone, PALS, liver, lungs, and bone marrow (Valladeau et al., 2002, Kamath et al., 2000). In the mouse, some Langerhans cells begin to express langerin at around 2 to 3 days after birth. By 10 days after birth, all Langerhans cells express langerin, and the intensity of expression reaches adult mouse levels by 3 weeks of age (Tripp et al., 2004). Unlike in humans, mouse Langerhans cells do not express CD1a (Valladeau et al., 2002).

10.8 Proliferation and Apoptotic Antigens

10.8.1 Proliferation Antigens

Evaluating cell proliferation in tissues of experimental animals is essential for toxicology and carcinogenesis studies and also for assessing the efficiency of cytotoxic and chemopreventive drugs in cancer research (Fong and Magee, 1999, Han et al., 2001). Proliferating cells can be detected in tissue sections by a number of methods. The gold standard has long been the in vivo labeling of DNA by the modified pyrimidine analogue BrdU, a halogenated derivative of thymidine (Gratzner, 1982, Alison, 1995). BrdU can be administered to laboratory animals via IP injection in pulse-label experiments or via osmotic pumps in continuous-label studies (Goldsworthy et al., 1991).

BrdU is readily incorporated into nuclei during the DNA synthetic phase (S phase) of the cell cycle and can be detected by immunohistochemical staining with

an anti-BrdU antibody. Immunohistochemical staining for BrdU has proved useful for identifying S-phase cells. However, this method has several disadvantages: (a) it requires injecting living animals and additional animal handling; (b) BrdU is also incorporated into cell DNA during DNA repair; and (c) BrdU is a mutagen and is thus not suitable for genetic toxicology studies.

For many years, in retrospective studies, the most common method of identifying proliferating cells in tissue sections without using BrdU has been by immunohistochemical detection of the proliferating cell nuclear antigen (PCNA, encoded by the *Pcna* gene), which was initially described by (Miyachi et al., 1978). PCNA is a well-conserved nuclear protein cofactor of DNA polymerases and other enzymes necessary for DNA synthesis (Kurki et al., 1986, Bravo and Macdonald-Bravo, 1987, Wood and Shivji, 1997). Expression of PCNA increases during the G1phase, peaks at the S phase, declines during G2/M phases and is absent in the G0 phase of the cell cycle. Cellular localization in normal and neoplastic cells can be nuclear, nuclear and cytoplasmic, or cytoplasmic, depending on the phase of the cell cycle and state of cell differentiation (Kamel et al., 1992, Foley et al., 1993, Bouayad et al., 2012). These immunostaining characteristics allow the identification of cells in all phases of the cycle (Foley et al., 1993). Detectable levels of PCNA can vary significantly among different cell types, between cells in the malignant vs. normal state, and depending on the fixatives and antigen-retrieval solutions used (Morris and Mathews, 1989, Hall et al., 1990, Coltrera and Gown, 1991, Schwarting, 1993, Scholzen and Gerdes, 2000). PCNA is also involved in DNA repair (Celis and Madsen, 1986, Toschi and Bravo, 1988, Shivji et al., 1992, Wood and Shivji, 1997), which suggests that PCNA is expressed by cells that are not cycling.

Another immunohistochemical technique that has found application in assessing cell proliferation is the detection of Ki67 (encoded by the *Mki67* gene). Ki-67 is a ubiquitous nuclear protein expressed in the G1, S, G2, and M phases of the cell cycle but not in the G0 phase (Gerdes et al., 1984, Gerlach et al., 1997). Monoclonal antibodies raised against the human Ki-67 protein often have a limited cross-species reactivity. The monoclonal antibody MIB-5 has been generated by using bacterially expressed parts of the human Ki-67 cDNA (Schluter et al., 1993, Gerlach et al., 1997). This antibody has the advantage of being able to react with the rodent-equivalent cell cycle–related nuclear protein. The percentages of anti-Ki67 and anti-BrdU immunostained cells correlate in rodent tissues (Ito et al., 1998, Birner et al., 2001, Enami et al., 2001, Fedrowitz et al., 2002). However, Ki67 is considered to be a more specific marker than BrdU or PCNA, because both BrdU and PCNA are incorporated in DNA repair, suggesting that these two proteins are expressed by cells that are not cycling.

Direct counting of mitotic cells in H&E-stained sections is an alternative to the previous three approaches for evaluating cell proliferation in tissue sections. However, direct counting of mitotic cells is time consuming and requires a highly skilled individual, and it is often difficult to distinguish between mitotic cells and cells undergoing pyknosis or apoptosis in tissue sections. Therefore, an immunohistochemical approach to detecting the expression of phosphohistone H3 (Histone H3, encoded by the *Hist1h3h* gene) is helpful in overcoming these obstacles (Hans and Dimitrov, 2001, Brenner et al., 2003, Muskhelishvili et al., 2003, Tsuta et al., 2011). Phosphohistone H3 is a mitosis marker closely associated with mitotic chromatin condensation in the late G2 and M phases of the cell cycle, and histone H3 is not phosphorylated during apoptosis.

10.8.2 Apoptosis Antigens

Caspase 3 (encoded by the *Casp3* gene) is a member of the apoptosis executioner group and is processed by any of the initiator caspases (8, 9, or 10) or granzyme B. The assay for caspase 3 is the most popular assay for apoptosis. Caspase 3 detection by immunohistochemical staining is a valuable tool for identifying apoptotic cells in FFPE tissue sections before and after the morphologic features of apoptosis occur. Antibodies to caspase 3 work well in mice, and the assay is more specific for apoptotic cells than are TUNEL stains, which may impart unusual artifacts and also stain necrotic cells.

When assaying cellular markers *Antigens* to measure either apoptosis or cell proliferation, it is important to keep in mind that specimen handling may affect the expression of these cellular antigens. Specifically, increases in the mean labeling indices of activated caspase 3, Ki67, and PCNA and a decline in the number of mitotic figures may occur if fixation is delayed (Scudamore, 2011). Therefore, it is essential that a tissue specimen be placed in the appropriate fixative as rapidly as possible after the animal is euthanized. Consequently, tissue fixation by whole-body perfusion is preferred over immersion fixation. Antibodies commonly used for assessing cell proliferation and apoptosis are listed in Table 10.8.

10.8.3 Cross-Species Immunoreactivity of Antibodies

Several antibodies to specific antigens work well in FFPE tissues of more than one species. For example, polyclonal anti-human TDT (Fig. 10.8), polyclonal anti-human CD3 (Fig. 10.12), polyclonal anti-human PAX5 (Fig. 10.13), and polyclonal human kappa light chains work in FFPE tissues from several species. However, most monoclonal antibodies work only in tissues of the species from which the antigen was obtained to make the antibodies. For examples of this, see the chart from (Dako, 2007). The vendor's data sheet for a commercially produced antibody should provide information on the species with which the antibody cross-reacts.

10.8.4 Rat, Monkey, Dog, and Pig Lymphoid Immunohistochemistry

Mouse IHC is commonly used in toxicology and medical research, whereas the IHC of other species is less commonly used in toxicology. The following publications report the use of antibodies for species other than mice that are used in toxicology but not in toxicologic pathology. A laboratory must check the datasheet for each antibody to see if information is provided on its applicability for FFPE tissue sections. Antibodies to rat leukocyte antigens that have been used for immunohistochemical analysis (van den

Table 10.8 Antibodies commonly used for detecting apoptosis and cell proliferation in mouse FFPE tissue

Antigen	Major cells expressing antigen	Antibody	Clone	Dilution	Source	Epitope retrieval
BRDU	S-phase cell cycle and in DNA repair	Rat anti-BRDU	Bu1/75 (ICR1)	150	Novus Biologicals	HIER ER1 (Leica, pH 6)
Caspase-3	Cell undergoing programmed cell death	Rabbit anti-human	n/a	500	Biocare Medical	HIER CC1 (Roche, pH 8)
Ki67	G1, S, G2, and M, phases of cell cycle	Rabbit anti-human	SP6	100	Thermo Fisher	HIER CC1 (Roche, pH 8)
pHistone H3	Mitosis in late G2 and M phases of cell cycle	Rabbit anti-human	n/a	200	Bethyl Laboratories	HIER Citrate (Invitrogen, pH 6)
PCNA	All phases of cell cycle and in DNA repair	Mouse anti-rat	PC10	1500	Dako	HIER Target Retrieval (Dako, pH 6)

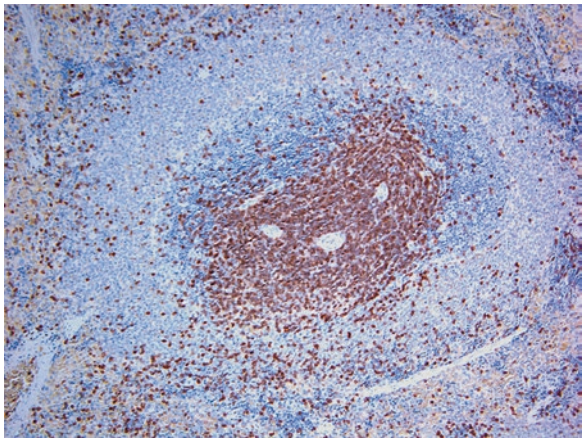

Fig. 10.12 Spleen of a young Sprague Dawley rat showing *white pulp* and *red pulp* T lymphocytes labeled with a polyclonal anti-human CD3 antibody (Chromogen: DAB)

Berg et al., 2001, Ward et al., 2006, Rehg et al., 2012) are listed in Table 10.4 and are readily available from various companies. Many of these antibodies work very well in FFPE tissue sections (Figs. 10.8, 10.12, and 10.13). Immunohistochemical analysis has also been described for tissues from monkeys (Ormerod et al., 1988, Li et al., 1993, Lappin and Black, 2003, Kap et al., 2009, Batchelder et al., 2014) and dogs; in the latter case for tumor diagnosis in particular but also for characterization of lymphoid cells (Burkhard and Bienzle, 2013). Immunohistochemical analysis of pig tissues is less common (Piriou-Guzylack and Salmon, 2008).

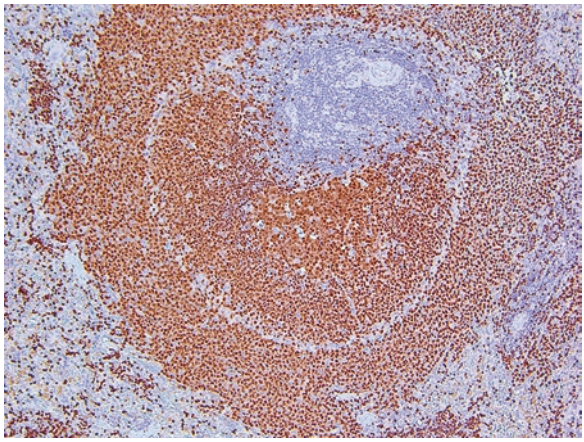

Fig. 10.13 Spleen of a young Sprague Dawley rat showing the nuclei of B lymphocytes of the germinal center, mantle zone, and marginal zone labeled with polyclonal anti-human PAX5 (Chromogen: DAB)

10.9 Examples of the Use of IHC for Evaluating the Hematolymphoid System

A review of the applications of IHC in toxicology and toxicologic pathology has been published (McDorman, 2013). The following are examples of IHC being used to address specific questions regarding cell lineage, cellular heterogeneity in organs and in hematopoietic malignancies, and the pattern of antigen expression in healthy and diseased tissues.

10.9.1 Assessment of the Normal Expression Patterns of Hematolymphoid Cells in Lymphoid Tissue and Bone Marrow

B lymphocytes, T lymphocytes, and macrophages have specific anatomical distribution patterns in the thymus, spleen, and lymph nodes. These patterns can be best appreciated by using antibodies specific to B cell, T cell, and macrophage/dendritic cell antigens. The thymic cortex and medulla, the two major anatomic compartments of the thymus, can be clearly defined with antibody to the T-lymphocyte CD3 antigen (Rehg et al., 2012). The periarteriolar lymphoid sheath of the white pulp of the spleen, which is populated predominantly with T lymphocytes, is clearly delineated by antibodies to the CD3, CD5, or CD43 antigens expressed on T lymphocytes (Fig. 10.14) (Rehg et al., 2012). Conversely, antibodies to the B-cell antigens CD45R/B220 or PAX5 will label the germinal centers, mantle cells, and marginal zone cells of the splenic follicles and the germinal centers and mantle cells of the lymph node follicles (Figs. 10.13 and 10.15). Antibodies to the macrophage MAC2 and F4/80 antigens can be useful in distinguishing different macrophage populations in lymphoid organs (Figs. 10.16 and 10.17).

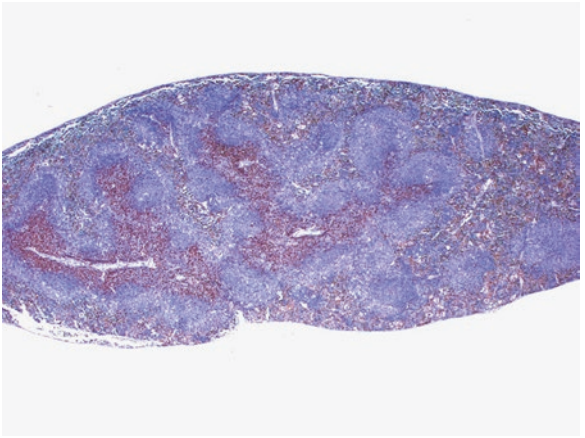

Fig. 10.14 Spleen of an adult FVB mouse showing the T lymphocytes in the PALS regions of the white pulp (follicles) with CD43 expression (Chromogen: DAB). Anti-CD3, CD5, and CD43 antibodies are very useful in delineating T-cell regions in lymphoid organs

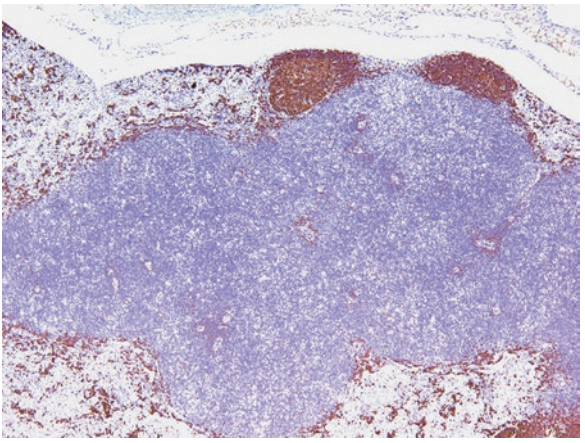

Fig. 10.15 Lymph node of an adult FVB mouse showing CD45R/B220 expression of B lymphocytes in the follicles and medullary cords (Chromogen: DAB). Anti-CD45R/B220 and PAX5 antibodies are useful in delineating the anatomical location and distribution of B lymphocytes, as well as the prominence and size of B-cell follicles in lymphoid organs

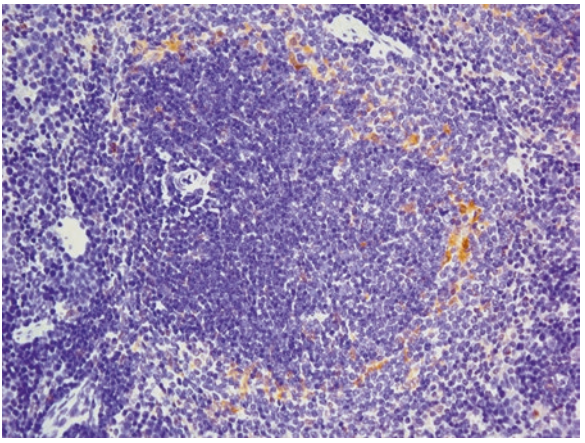

Fig. 10.16 Spleen of an adult FVB mouse showing prominent MAC2 expression in the macrophages of the marginal-zone sinus. Only a few macrophages in the marginal zone and red pulp express MAC2 (Chromogen: DAB)

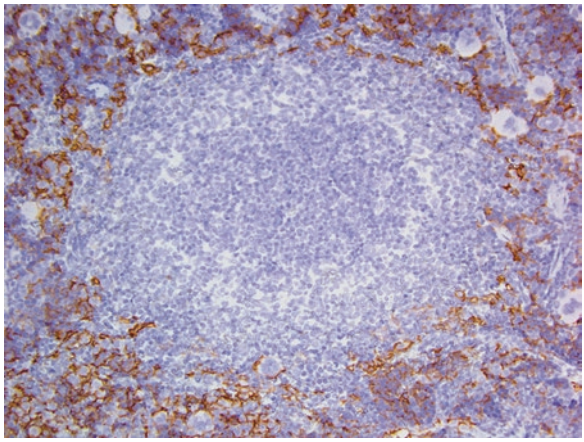

Fig. 10.17 Spleen of an adult FVB mouse showing extensive F4/80 expression in the red pulp macrophages and not in the white pulp. Note that the marginal-zone sinus macrophages do not express F4/80 (Chromogen: DAB)

10.9.2 Development of Abnormal Patterns of Antigen Expression in Lymphoid Cells and Tissues after Exposure to Toxins and Toxicants and in Neoplasia

Toxins, toxicants, and immunocytes may affect the lymphoid and hematopoietic systems, causing cell degeneration and cell death followed by hyperplasia and even neoplasia in the various lymphoid tissues. These effects may be recognized by the loss of normal tissue anatomy and histology, the loss of specific cell types, and an increase in other cell types, especially inflammatory cells that are not normally present in those tissues, and all the other changes associated with histopathologic lesions. Regenerative changes may occur and are characterized by changes in the antigen expression in various cells and in cell proliferation antigens. The toxins, toxicants, and immunocytes may also affect cells in other tissues via infiltrating inflammatory cells. The cellular lineage of the cells involved in these conditions can be characterized by immunohistochemical analysis (Figs. 10.18, 10.19, and 10.20).

In Fig. 10.21, an H&E-stained section shows an inflammatory cell infiltrate in the bone marrow of a 30-month-old male B6,129 mouse with a toxemia. The infiltrate consists of cells with abundant pink and foamy pink cytoplasm and central or slightly eccentric nuclei with condensed chromatin. The histologic features suggest that the infiltrating cells are macrophages, plasma cells, or monocytoid lymphocytes. Using the immunohistochemical antibody panel for CD3, PAX5, MAC2, IBA1, CD138, and IRF4 revealed that the cells expressed CD138 and IRF4 (Figs. 10.22 and 10.23) and were negative for MAC2, IBA1, CD3, and PAX5.

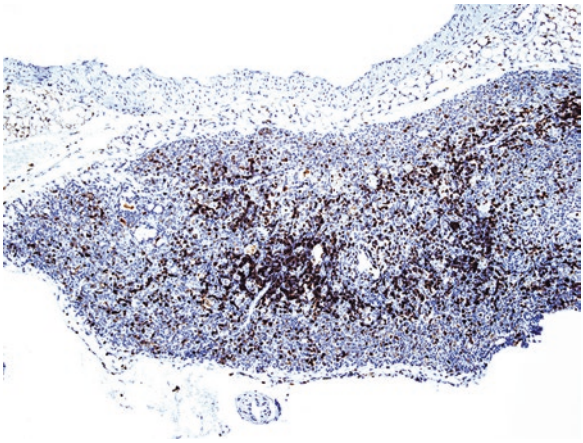

Fig. 10.18 Thymus of a mouse given a lymphocyte toxicant 48 h previously, showing depletion of CD3+ lymphocytes (Chromogen: DAB)

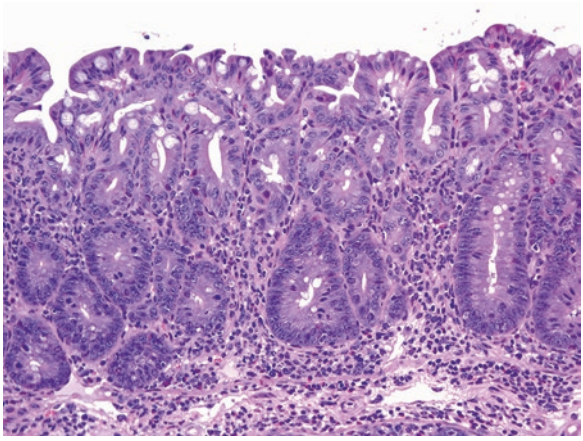

Fig. 10.19 Colon with graft-versus-host disease in an irradiated C57BL/6 mouse given BALB/c mouse T lymphocytes. The colonic crypts are elongated, and there is a mixed inflammatory-cell infiltrate in the mucosa and submucosa that consists of lymphocytes and eosinophils

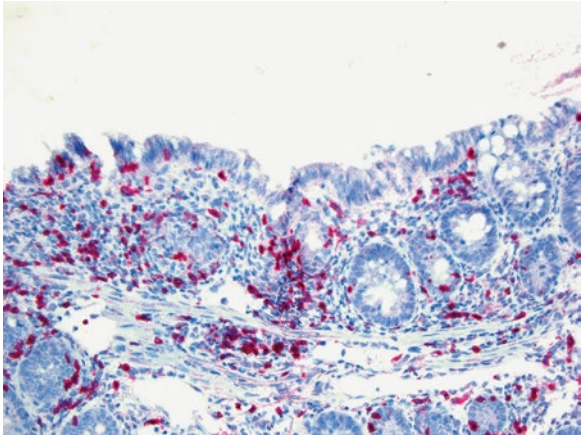

Fig. 10.20 The inflammatory cells shown in Fig. 10.19 consist predominately of lymphocytes expressing CD8 (Chromogen: Fast Red.)

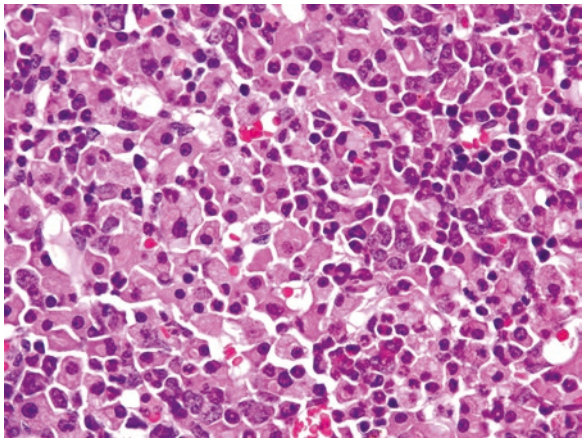

Fig. 10.21 Bone marrow of a 30-month-old male B6, 129 mouse with multiple foci of numerous cells having central and eccentric nuclei and abundant foamy pink cytoplasm

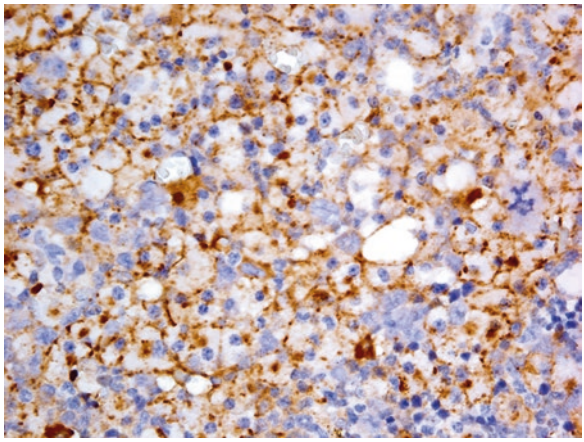

Fig. 10.22 The foamy cells in Fig. 10.21 express membrane CD138, which suggests they are plasma cells (Chromogen: DAB)

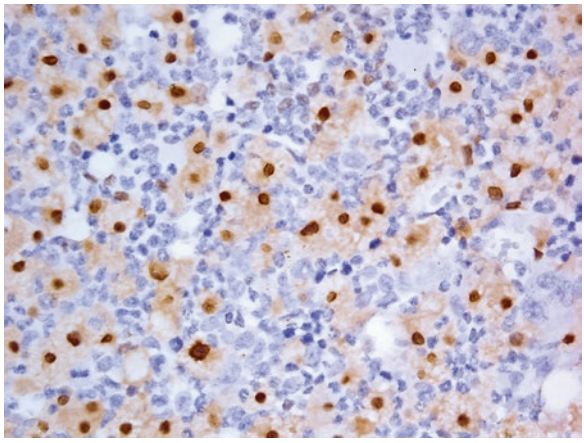

Fig. 10.23 The foamy cells in Fig. 10.21 also express nuclear and cytoplasmic IRF4. Their expression of both IRF4 and CD138, but not F4/80, Mac 2, CD3 or PAX5, is consistent with the foamy cells being plasma cells (Chromogen: DAB)

10.9.3 Loss of an Antigen Commonly Associated with a Cell

Antigens can be lost from cells that normally express them when they are involved in various non-neoplastic and neoplastic lesions, necessitating using a panel of antibodies directed against more than one of the antigens that a specific cell may express.

The splenic marginal zone cells normally express the pan-B-cell antigen CD45R/B220. In Fig. 10.24, an H&E-stained section of a splenic marginal zone lymphoma in an adult B6,129 mouse shows a mixture of small and large cells with abundant pink cytoplasm that do not express CD45R/B220 (Fig. 10.25) but do express nuclear Pax5 (Fig. 10.26), confirming that the lymphoma is of B-cell lineage. The cytologic morphol-

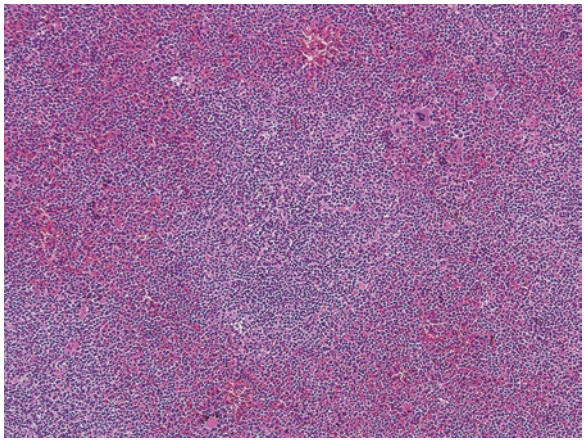

Fig. 10.24 Spleen from an adult B6, 129 mouse showing expansion and convergence (bridging) of marginal zone cells, suggestive of a marginal-zone lymphoma

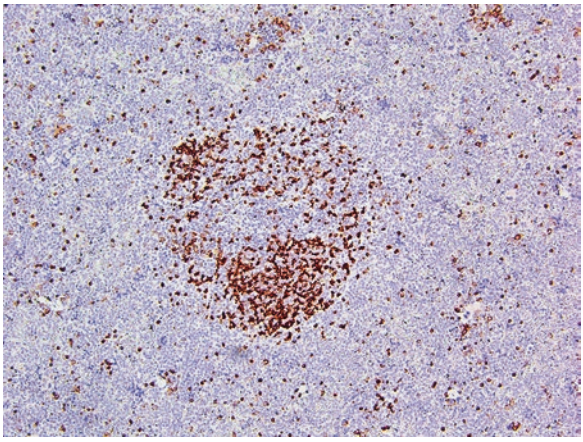

Fig. 10.25 The lymphoma cells present in Fig. 10.24 do not show expression of CD45R/B220 after antibody labeling. The follicular mantle zone cells do show expression of CD45R/B220, but the continuity of this zone has been disrupted with the CD45R/B220-negative lymphoma cells. Marginal zone lymphocytes normally express CD45R/B220, but approximately 20% of mouse marginal zone lymphomas do not express it (Chromogen: DAB)

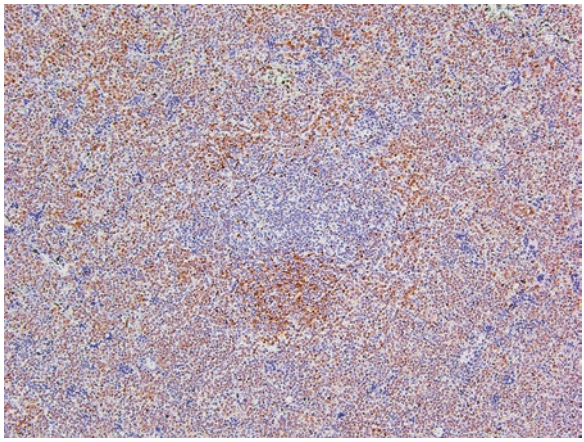

Fig. 10.26 The lymphoma cells in Fig. 10.24 involving the red pulp, marginal zone, and mantle zone, which were shown in Fig. 10.25 to be negative for CD45R/B220 expression, do show nuclear expression of PAX5, which is consistent with a marginal zone lymphoma (Chromogen: DAB)

ogy, growth pattern, and immunohistochemical profile are consistent with a marginal zone lymphoma. It should be noted that approximately 20% of splenic marginal zone lymphomas in the mouse do not express CD45R/B220 (Rehg and Sundberg, 2008).

It is also not uncommon for T-cell lymphomas to lose one or more of the four pan-T-cell antigens (CD2, CD3, CD5, and CD7). Antigen loss is defined by some as staining of less than 50% of the total T cells. Figure 10.27 shows an H&E-stained section of mouse skin with peripheral T-cell lymphoma cells involving the epidermis and dermis. Figure 10.28 shows that the neoplastic T lymphocytes have lost their expression of CD5.

10.9.4 Alterations in the Number or Distribution of Hematolymphoid Cells in a Tissue

It is not uncommon for hyperplasia to be associated with any cell of the hematolymphoid system, as in marginal zone lymphocyte hyperplasia where an animal has particulate antigen in its circulation system. It is also not uncommon for plasma cell hyperplasia to occur in the bone marrow when there is chronic antigen stimulation, as occurs with abscesses and tumors. Bone marrow plasma cell hyperplasia may not be appreciated unless the bone marrow is labeled with antibody to one or more of the plasma cell antigens discussed. The H&E-stained section in Fig. 10.29 shows bone marrow plasma cell hyperplasia in an adult B6,129 mouse with a chronic preputial gland abscess. In the H&E-stained section, the extent of the plasma cell hyperplasia is not apparent. However, when the tissue is labeled with anti-IRF4 antibody (Fig. 10.30) and CD138, the extent of the plasma cell increase in the bone marrow can be appreciated. Furthermore, bone marrow hyperplasia will need to be differentiated from a plasmacytoma. The immunostaining for IRF4 (Fig. 10.30) further illustrates how the plasma cells are intermingled with myeloid and erythroid cells and do not form a solid sheet typical of a plasmacytoma.

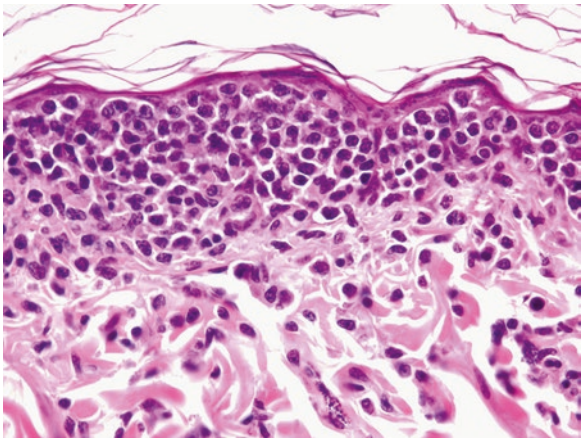

Fig. 10.27 Skin from a15-month-old male B6,129 mouse with infiltrating T-cell lymphoma cells in the dermis and epidermis. The epidermis is expanding in size because of the lymphoma infiltrate

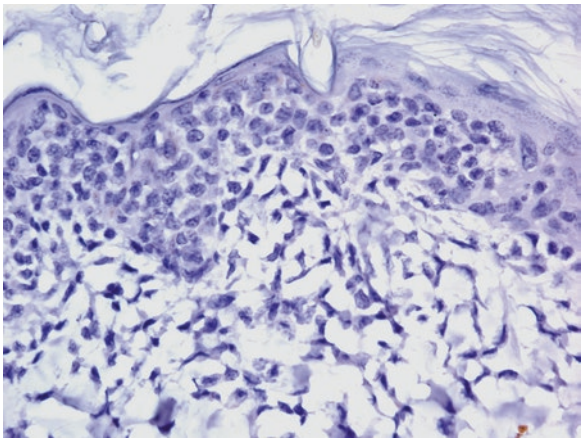

Fig. 10.28 The T-lymphoma cells shown in Fig. 10.27 lack CD5. Unlike normal T lymphocytes, it is not uncommon for T-cell lymphomas to lose one or more of the four pan–T-cell antigens: CD2, CD3, CD5, and CD7 (Chromogen: DAB)

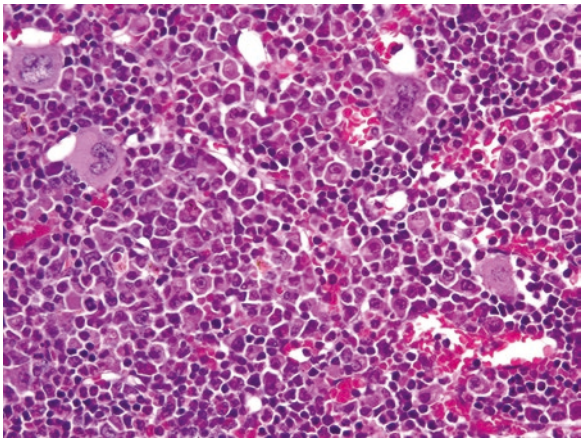

Fig. 10.29 Bone marrow from a 24-month-old male B6,129 mouse with a cutaneous abscess. The bone marrow has a mixed population of megakaryocytes, immature granulocytes, and immature and mature erythroid cells, along with a scattering of cells with a pale paranuclear Golgi halo that is suggestive of plasma cells

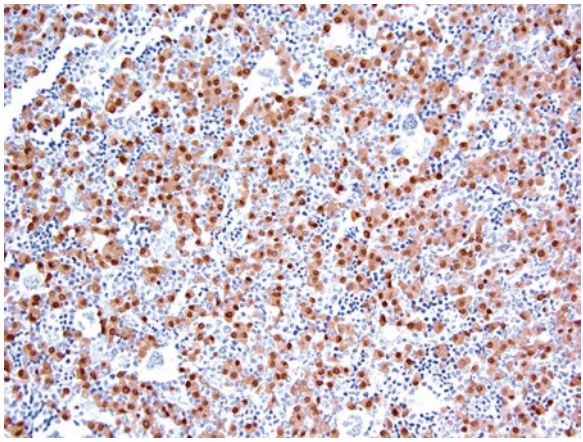

Fig. 10.30 Labeling the bone marrow in Fig. 10.29 with anti-IRF4 antibody reveals an increased number of cells with nuclear and cytoplasmic expression of IRF4. The cells with IRF4 immunoreactivity are uniformly scattered among myeloid and erythroid cells, consistent with plasma-cell hyperplasia (Chromogen: DAB)

10.9.5 Changes in the Expression of Antigens Associated with a Cell

Neutrophils often occur in large numbers in the spleen, and they may be associated with splenic physiologic myeloid hyperplasia in response to an increased production of granulocytes, or they may be a reactive inflammatory infiltrate. A major differentiating factor between these two host reactions is whether the neutrophil is in an activated state. Unlike resting neutrophils, activated neutrophils show strong cytoplasmic expression of iNOS2 (Richter et al., 1999, McCafferty et al., 1999, Morton et al., 2008). Figure 10.31 shows resting neutrophils with weak low levels of iNOS2, and Fig. 10.32 shows activated neutrophils with strong high levels of iNOS2. The enhanced expression of iNOS2 by the neutrophils is consistent with their being activated and of an inflammatory nature.

10.9.6 Identification of Hematolymphoid Cells Associated with an Inflammatory Lesion

In an inflammatory cell reaction, the antigen expression on macrophages depends on their microenvironment. Macrophage activation as a feature of cellular immunity to intracellular infectious pathogens depends on the products of specifically activated T-helper lymphocytes and NK cells. Mouse macrophages associated with inflammatory reactions in response to infectious agents are activated through the classic Th1 pathway or the alternative Th2 pathway and are referred to as M1 or M2 macrophages, respectively. M1 macrophages express high levels of iNOS2 and downregulate F4/80 expression upon activation by the CD4 TH1 proinflammatory cytokines IFNγ and TNFα. M2 macrophages express high levels of arginase, YM1, FIZZI, and mannose receptor (MRC1) upon activation by the CD4 TH2 cytokines IL4 and IL13 (Nair et al., 2003, Gordon and Martinez, 2010).

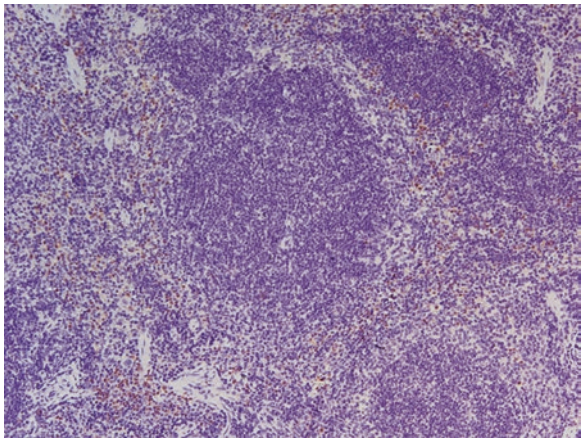

Fig. 10.31 A mouse spleen with little to no cytoplasmic expression of iNOS2 in the red pulp neutrophils, typical of a healthy mouse (Chromogen: DAB)

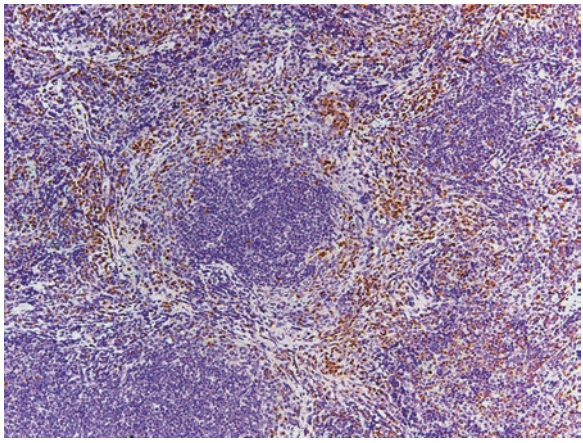

Fig. 10.32 A mouse spleen with red pulp neutrophils expressing high levels of cytoplasmic iNOS2, typical of activated neutrophils (Chromogen: DAB)

In Fig. 10.33, an H&E-stained lung section illustrates a pulmonary lymphogranulomatous lesion with lymphocytes surrounding epithelioid macrophages that express MAC2 and iNOS2 (Fig. 10.34 and Fig. 10.35). The immunophenotype of the lesion is consistent with classic CD4 Th1–pathway macrophage activation.

To understand the pathogenesis of an infectious disease, it is essential to understand the host's respond to the pathogen. Figures 10.36, 10.37, 10.38, 10.39, and 10.40 show a typical inflammatory phenotype of viral infections in the BALB/c mouse. Figure 10.36, shows an H&E-stained section of lung from a BALB/c mouse with a subacute inflammatory-cell reaction to influenza virus infection. The inflammatory-cell reaction consists of CD3+ lymphocytes (Fig. 10.37), Ly6G+ neutrophils (Fig. 10.38), and F4/80-positive macrophages (Fig. 10.39) that express arginase (Fig. 10.40), consistent with an M2 phenotype of the alternative CD4 Th2 macrophage activation pathway.

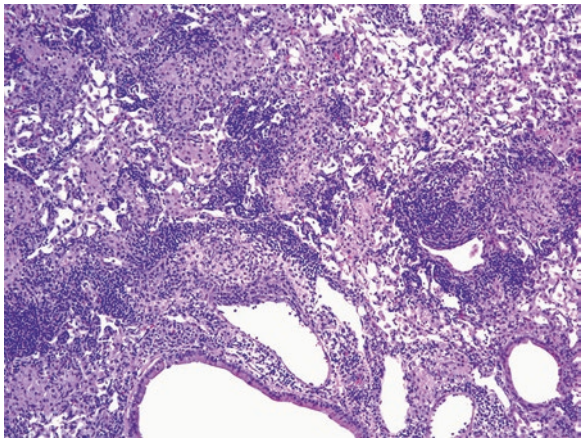

Fig. 10.33 Lung from an adult C57BL/6 mouse with a lymphogranulomatous pneumonia containing small and large foci of mononuclear cells with pink cytoplasm (macrophages) associated with small lymphocytes

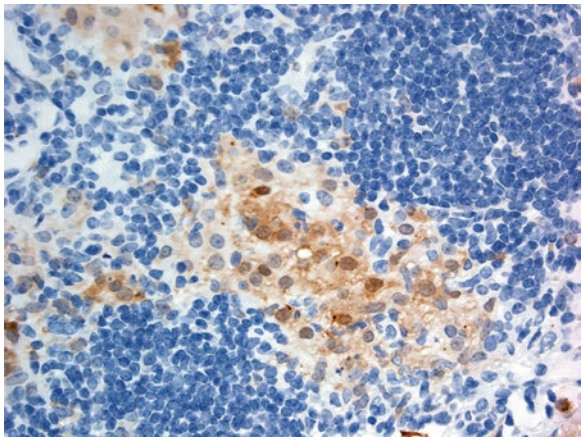

Fig. 10.34 The cells with pink cytoplasm in the mouse lung in Fig. 10.33 express MAC2. Chromagen: DAB

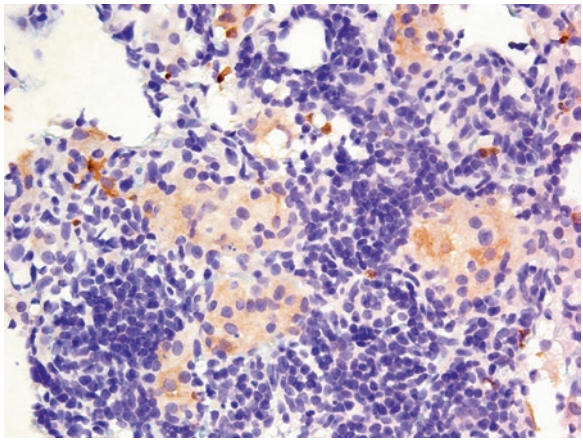

Fig. 10.35 The mononuclear cells expressing MAC2 in Fig. 10.34 also express iNOS2, which is characteristic of M1 macrophages activated through the CD4 Th1 classic pathway (Chromogen: DAB)

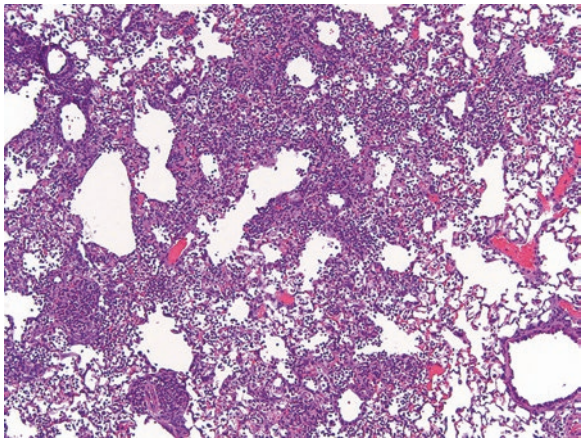

Fig. 10.36 Lung from an 8-week-old BALB/c mouse infected with influenza virus showing pneumonia with inflammatory cells in the alveoli and interstitium

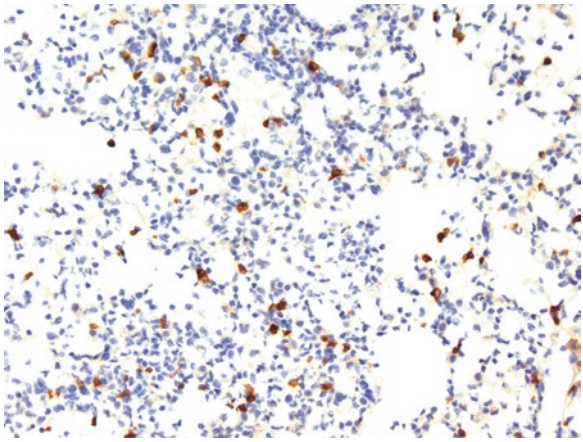

Fig. 10.37 In the mouse lung in Fig. 10.36, some of the inflammatory cells express CD3, consistent with the cells being T lymphocytes (Chromogen: DAB)

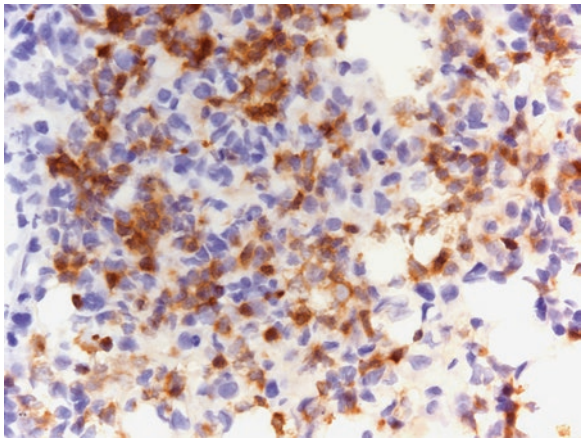

Fig. 10.38 Other cells associated with the inflammatory-cell infiltrate in the lung of Fig. 10.36 show cytoplasmic expression of Ly6G, which is characteristic of neutrophils (Chromogen: DAB)

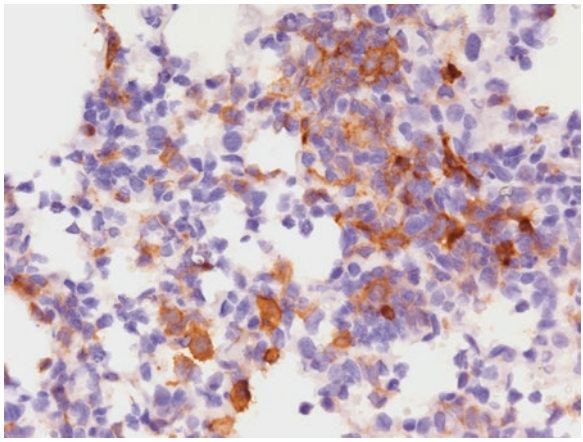

Fig. 10.39 The large mononuclear cells in the mouse lung inflammatory-cell infiltrate in Fig. 10.36 have pale cytoplasmic and dark membrane expression of F4/80 (Chromogen: DAB)

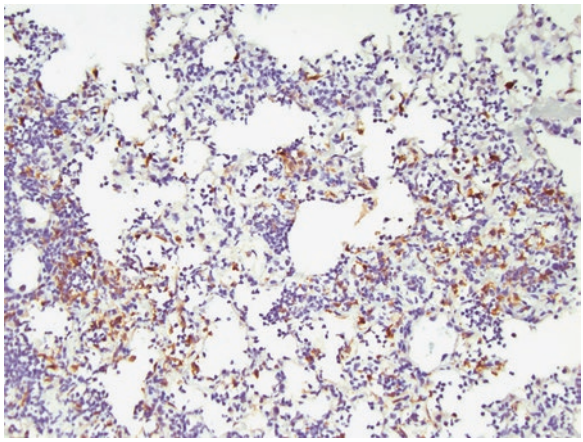

Fig. 10.40 The mononuclear cells in Fig. 10.39 that express F4/80 also express cytoplasmic arginase 1, which is characteristic of M2 macrophages activated through the CD4 Th2 alternative pathway (Chromogen: DAB)

10.9.7 Cell Proliferation Markers

Accurate assessments of the expression of markers that are specific for aspects of cellular proliferation, mitotic division, and apoptosis in preclinical systems are essential for the correct interpretation of test article effects in toxicology, carcinogenesis, and preclinical efficacy studies.

Figure 10.41 shows an H&E-stained section of a plasma cell tumor from a B6,129 male mouse. Figures 10.42 and 10.43 show representative images of sections labeled for the proliferation antigens Ki67 and PCNA, respectively. The large number of tumor cells (>90%) with positive nuclear labeling is consistent with their having a high proliferation rate and with the majority of the tumor cells being in various stages of the cell cycle. In parallel with an increase in proliferation, many tumor cells also show labeling for nuclear pHistone 3 (Fig. 10.44), which further indicates that the tumor has a high

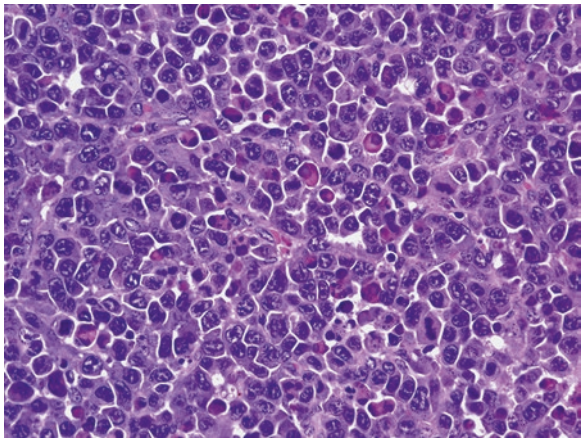

Fig. 10.41 An H&E-stained section of a mitotically active immature plasmacytoma with a scattering of apoptotic cells

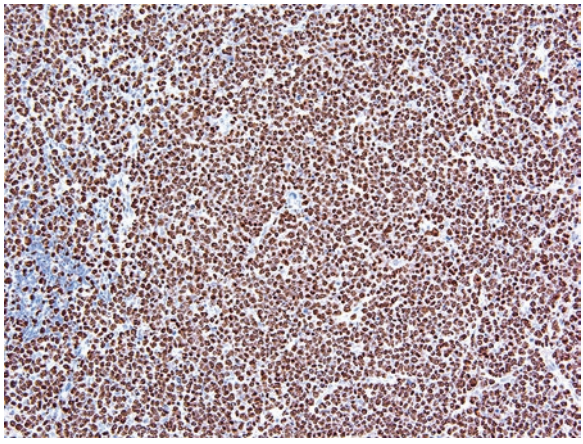

Fig. 10.42 The plasmacytoma in Fig. 10.41, when labeled with anti-Ki67 antibody, shows nuclear expression of Ki67 in more than 90% of the tumor cells, indicative of the tumor cell population being in various stages of the cell cycle and highly proliferative (Chromogen: DAB)

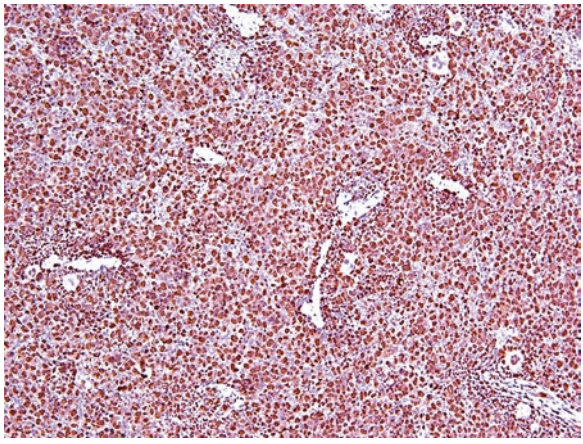

Fig. 10.43 When the tumor cell population in Figs. 10.41 and 10.42 is labeled with anti-PCNA antibody, cells are shown to express nuclear as well as cytoplasmic PCNA, consistent with a high percent of the tumor cells being in the G2 and M phases of the cell cycle (Chromogen: DAB)

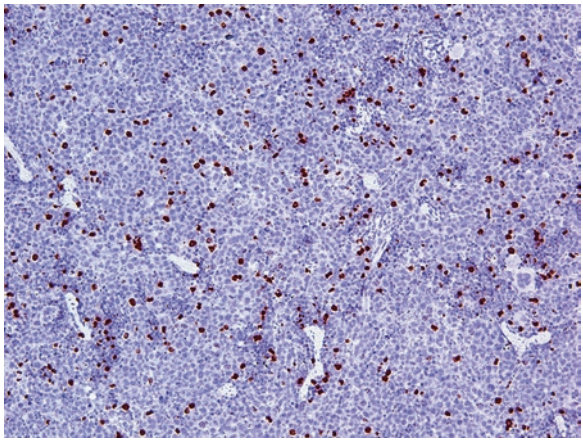

Fig. 10.44 The plasmacytoma in Fig. 10.41 also has a large number of cells expressing pHistone 3 in the nucleus, indicating that the tumor has a high mitotic rate (Chromogen: DAB)

mitotic rate. The caspase 3 staining (Fig. 10.45) shows that there is also scattered cellular apoptosis. Together, the results obtained with this panel of markers indicate that the catabolic state of the tumor is less than the anabolic state, especially when the degree of apoptosis is compared with the proliferation and mitotic indices. Manual counts of tumor regions or automated whole-slide analyses can be performed to assess these parameters either singly or jointly. Manual counting is very time consuming and has the potential for a greater degree of both inter- and intra-observer variability. A more precise and consistent quantitation of these indices can be reasonably achieved by using traditional immunohistochemical labeling methods and by developing and training computer-based algorithms that are designed to help address the specific study questions.

10.9.8 Diagnosis of Hematolymphoid Disorders

Conventional morphology remains the gold standard for evaluating hematolymphoid proliferations in paraffin-embedded tissue. However, IHC is playing an ever greater role in the diagnosis and classification of these lesions (Morse et al., 2002, Kunder et al., 2007, Swerdlow, 2008). Myeloid and lymphoid proliferations manifest antigen expressions that typify stages of their development and maturation. Consequently, the proliferative cells may express antigens of early precursor cells, various intermediate developmental stages, or late mature cell stages. By using a panel of antibodies to specific cellular lineages, it is often possible to determine whether a proliferation is of the myeloid, erythroid, megakaryocytic, lymphoid, plasma cell, histiocytic, or mast cell lineage.

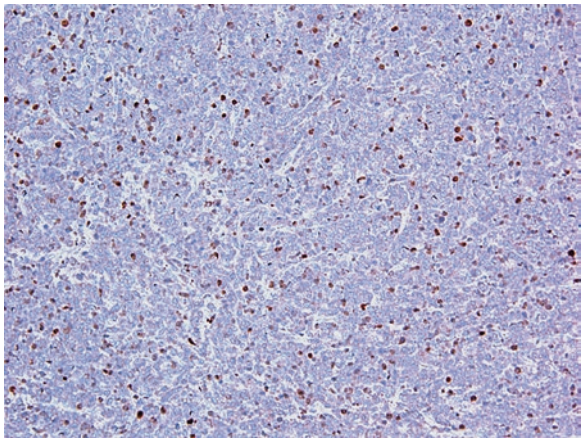

Fig. 10.45 The plasmacytoma in Fig. 10.41 has a scattering of tumor cells that express caspase 3, which is consistent with the cells being apoptotic (Chromogen: DAB)

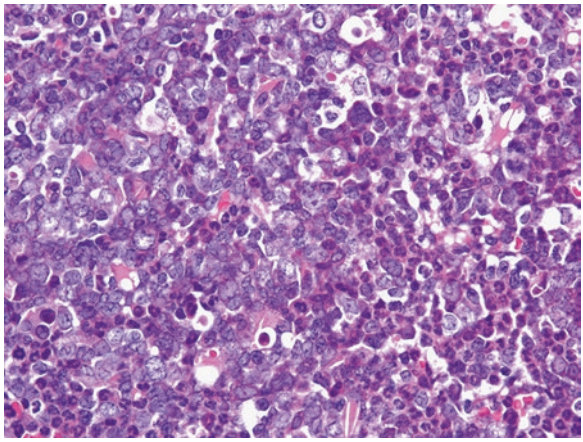

Fig. 10.46 Bone marrow from an adult B6, 29 mouse showing replacement of the normal trilineage bone marrow population by a monomorphous population of cells with blastic features

10.9.9 Diagnosis of Myeloid Proliferations

The most useful antigen markers for myeloid proliferation are CD34, CD43, CD45, CD68, MPO, lysozyme, GATA1, glycophorin A, TER119, and von Willebrand factor (Factor VIII).

Figures 10.46 through 10.49 show examples of myeloid leukemia in an adult B6,129 mouse. Infiltrates of a proliferative population of monomorphic cells with morphologic features of blasts and immature myeloid forms are seen in the bone marrow (Fig.10.46), liver, and spleen. With there being many blast cells, a lymphoblastic proliferation must be ruled out. Therefore, the tissues are labeled with antibodies to the myeloid antigens CD34, CD43, CD68, CD117, lysozyme, MPO, and GATA l and the lymphoid antigens CD3 and PAX5 to determine whether the proliferation is of myeloid or lymphoid lineage. The immunohistochemical analysis reveals that the cells express CD34 (Fig. 10.47), CD43, lysozyme (Fig. 10.48), and MPO (Fig. 10.49), but not CD68, CD3 or PAX 5, which is consistent with an acute myeloid leukemia.

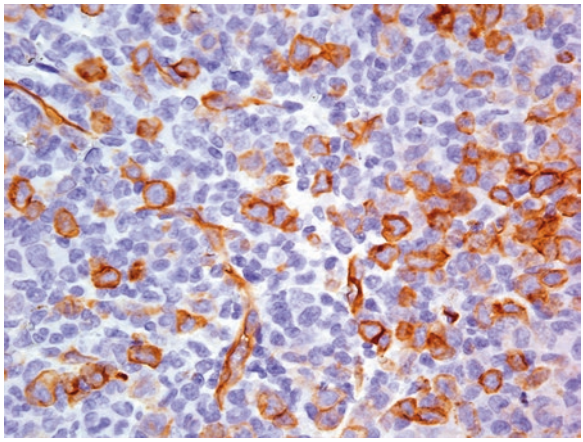

Fig. 10.47 Some cells in the cellular proliferation in Fig. 10.46 express CD34 (Chromogen: DAB)

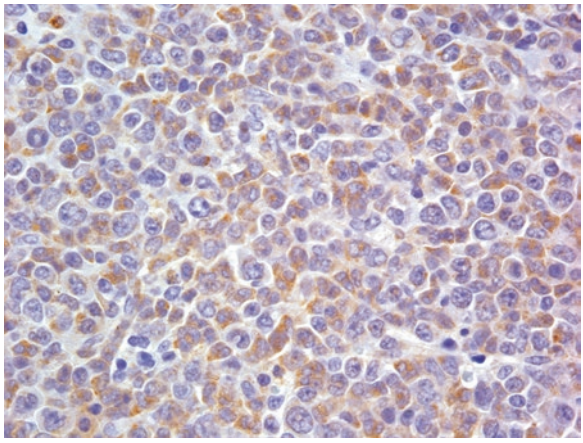

Fig. 10.48 A high percentage of the cells in the cellular proliferation in Fig. 10.46 express cytoplasmic lysozyme (Chromogen: DAB). Immature cells with ring (donut)-shaped nuclei are present in the proliferation

Figures 10.50 and 10.51 show an example of monocytic leukemia. The proliferation involves the spleen, lymph nodes, liver, lung, and bone marrow of a 7-month-old B6,129 mouse. The proliferation consists of large cells of variable size with blast features and donut-shaped nuclei (Fig.10.50). The tumor tissue was labeled with antibodies to CD34, CD43, MPO, lysozyme, CD68, and GATA1. The tumor cells were not labeled for CD34, CD43, GATA1, or MPO. The presence of donut-shaped nuclei and tumor cells expressing CD68 and lysozyme but not MPO suggested that the proliferation was of monocytic-macrophage lineage. However, the possibility that the tumor was of granulocytic lineage could not be completely ruled out, because the immunohistochemical characterization of mouse hematopoietic leukemias has not been sufficiently studied. Therefore, the tumor tissue was subsequently analyzed with antibodies to IBA1, MAC2, and F4/80.

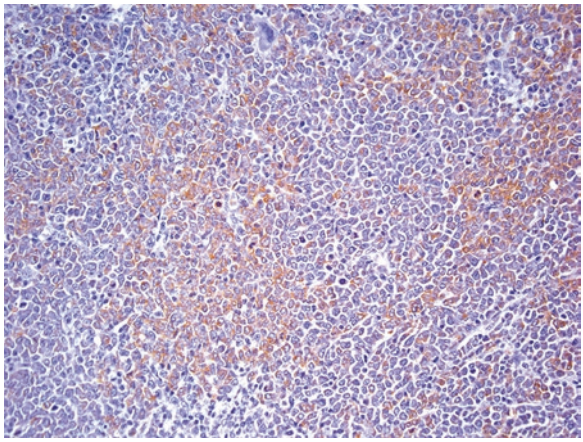

Fig. 10.49 Nodular areas of blast cells in Fig. 10.46 express cytoplasmic myeloperoxidase (Chromogen: DAB)

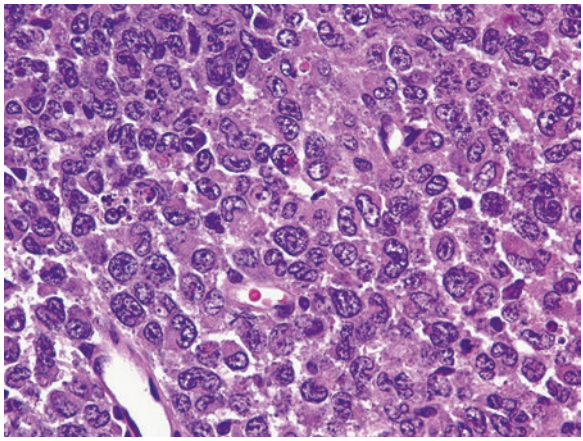

Fig. 10.50 Bone marrow from a 9-month-old male B6,129 mouse showing replacement of the normal trilineage bone marrow population by cells with ring (donut)-shaped nuclei and other immature nuclear forms

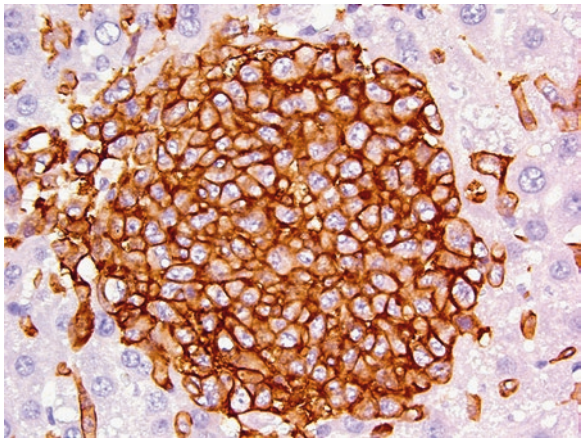

Fig. 10.51 Liver nodules consisting of cells with similar morphologic features to cells in the bone marrow in Fig. 10.50 express IBA1, consistent with the leukemia being of a monocytic lineage (Chromogen: DAB)

The tumor cells were labeled by the antibodies to IBA1 (Fig. 10.51) and MAC2, but not by the antibody to F4/80. In addition to granulocytes, some immature stages of mouse monocytes also have a donut shape (Biermann et al., 1999). Unlike macrophages, monocytes either do not express or only very weakly express F4/80 (Francke et al., 2011). Therefore, it was concluded that the cytologic morphology and immunophenotype profile was most consistent with a monocytic leukemia.

Figures 10.52 through 10.54 show an example of erythroid leukemia. The proliferation involves the spleen, liver, lung, and bone marrow of an adult B6,129 mouse and consists of cells of variable size with blast features and small cells with condensed chromatin and pale pink cytoplasm that are suggestive of erythroid differentiation (Fig. 10.52). Using the myeloid panel of antibodies to CD34, CD43, CD68, MPO, and GATA1 revealed that the leukemia cells were immunoreactive for GATA1 (Fig. 10.53), which suggested the cells were of erythroid or megakaryocytic lineage. Subsequent labeling of the leukemia tissue with antibodies to glycophorin A and CD41 revealed that the leukemia cells expressed glycophorin A (Fig. 10.54) and not CD41, which is consistent with an erythroid leukemia.

Figures 10.55 and 10.56 illustrate a megakaryocytic leukemia proliferation in an adult B6,129 mouse. The proliferation involves the spleen, liver, and bone marrow, with cells that have round and irregular nuclei admixed with binucleated and multinucleated cells that have blastic nuclear chromatin features and sparse cytoplasm (Fig. 10.55). Using a myeloid panel of antibodies to CD34, CD43, CD68, MPO, GATA1, CD41 and von Willebrand) revealed that the leukemia cells expressed nuclear GATA1 and cytoplasmic von Willebrand factor and CD41 (Fig. 10.56). The

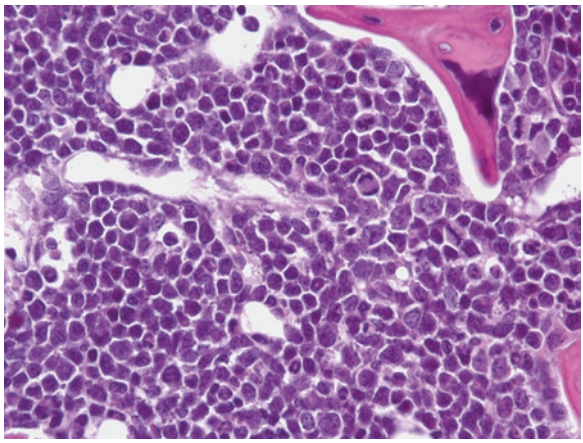

Fig. 10.52 Bone marrow from an adult B6, 129 mouse showing replacement of the normal trilineage bone marrow population by a high mitotically active population consisting primary of cells with blastic nuclei and some small cells having condensed chromatin and a faint pink cytoplasm

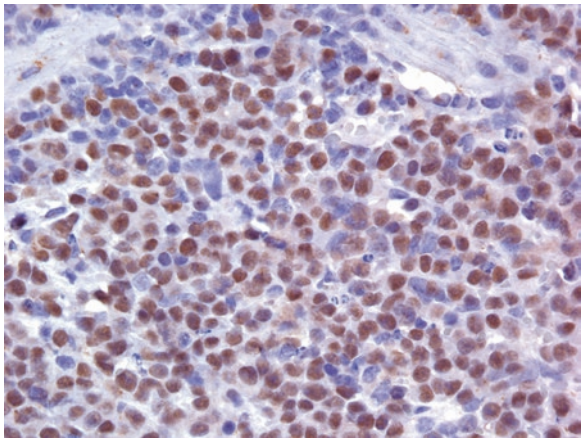

Fig. 10.53 The population of cells in Fig. 10.52 shows nuclear expression of GATA1, which is consistent with erythroid or megakaryocytic lineage (Chromogen: DAB)

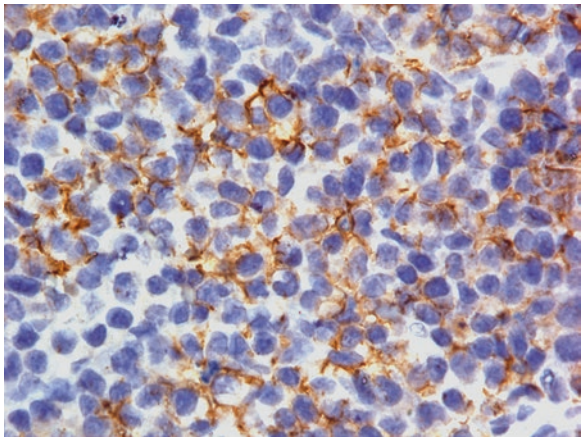

Fig. 10.54 Many of the cells in Fig. 10.52 also show membrane expression of glycophorin A (CD 235a). The cell morphology and immunohistochemical profile in Figs. 10.52, 10.53, and 10.54 are consistent with an erythrocytic leukemia (Chromogen: DAB)

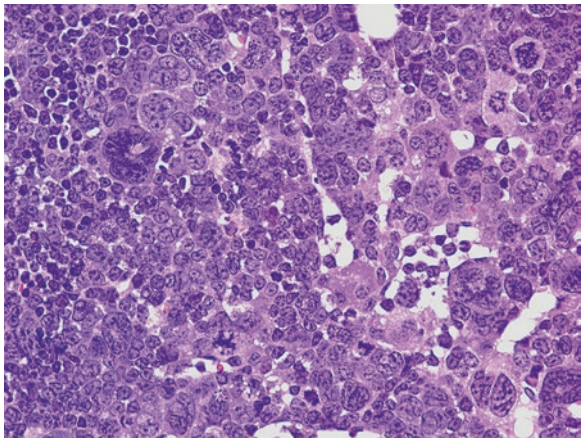

Fig. 10.55 Bone marrow from an adult B6, 129 mouse showing replacement of the normal trilineage bone marrow population by a mitotic active population consisting of small cells with blastic features, multiple cells with a lobated nucleus and cells with variable sized atypical nuclei

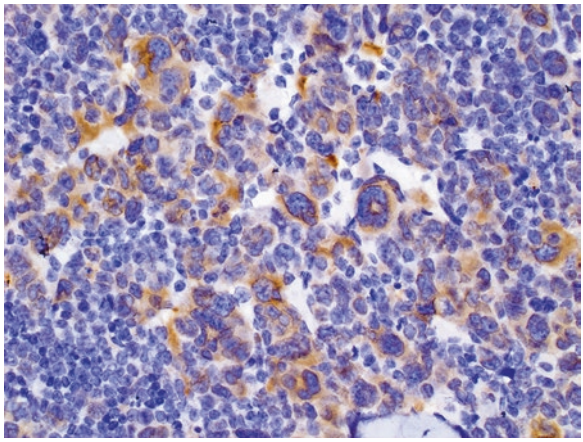

Fig. 10.56 Some of the cells with atypical and lobated nuclei featured in Fig. 10.55 show cytoplasmic expression of CD41. The small cells with blastic features expressed GATA (not shown). The cell morphology and immunohistochemical profile of the proliferation is consistent with a megakaryocytic leukemia (Chromogen: DAB)

cells were not labeled by any of the other four myeloid panel antibodies or by antibodies to PAX5 or CD3. Therefore, the immunophenotype is compatible with a megakaryocytic leukemia.

10.9.10 Diagnosis of Lymphoma

Lymphoma represents a large category of lymphoid neoplasms, and it is beyond the scope of this review to address the various classifications and their immunophenotypes. For lymphoma classification in the mouse, see the reviews by Morse et al. (2002) and Rehg et al. (2015). Here, we illustrate the principles of diagnosing a subclass of lymphoblastic lymphoma by immunohistochemical analysis. This is just one biomarker-based method for diagnosing lymphomas (Schafernak et al., 2014); clonality assays should be used, when possible, if a chemical induces a lymphoproliferative disorder that may be lymphoma (Ward et al., 2012). PCR analysis to show gene rearrangements has not been used successfully to demonstrate clonality in mice or rats, so Southern blotting and immunohistochemical analysis are the diagnostic methods of choice in those species. However, PCR does work for diagnosis in humans, dogs, and monkeys (Schafernak et al., 2014, Burkhard and Bienzle, 2013).

10.9.11 IHC Criteria for B-Cell Malignancy

CD43 has not been shown to be expressed in mouse B lymphocytes other than immature B cells and plasma cells. Therefore, the expression of CD43 in all the cells of a B-cell proliferation is highly suggestive of a B-cell neoplasm. Frequently, one may observe two levels of staining for CD43 in a proliferation; the

non-neoplastic T cells will have the strongest intensity of staining, whereas the neoplastic B cells will have weaker stain intensity.

In the authors' experience, CD5 expression is only rarely detected in B-cell proliferations by immunohistochemical staining. Therefore, the immunohistochemical detection of strong CD5 expression on a B-cell proliferation in a mouse should be taken as strong evidence of a neoplastic B-cell proliferation.

The number of interfollicular B immunoblasts and other interfollicular B-lymphoid cells does not exceed the number of T cells. Therefore, the presence of sheets of B cells involving the interfollicular areas strongly favors the diagnosis of a B-cell lymphoma.

In addition to the above, the following example illustrates some of the criteria to be considered when a proliferation is composed of cells with a lymphoblastic morphology that involves multiple lymphoid tissues. The tumor cells in this example were not immunoreactive for CD45R/B220 or CD3, and the proliferation was initially diagnosed as lymphoblastic lymphoma, not otherwise specified (NOS). In a retrospective lymphoma study, the lymphoma cells were labeled for CD117 (KIT), CD43, TDT, and PAX5, but not for cytoplasmic μIgM heavy chains (Rehg and Sundberg, 2008). The immunoreactivity of the lymphoblasts for CD117 (Fig. 10.57) and PAX5 (Fig. 10.58) but not for μIgM is consistent with the lymphoma being a pro-B–lymphoblastic lymphoma, based on the antigen expression scheme of B-cell ontogeny in Table 10.5.

10.9.12 IHC Criteria for T-Cell Malignancy

The loss of CD5 in a T-lymphoblastic proliferation that is positive for CD3 or CD90 is highly suggestive of the proliferation being a T-cell lymphoma (Rehg et al., 2015).

Generally, mature T cells are TDT− and express either CD4 or CD8. Therefore, the detection of either a double-negative (CD4−CD8−) or a double-positive (CD4+CD8+) population may be considered evidence of a T-cell lymphoma.

Figures 10.27, 10.28, and 10.59 show a representative example of a proliferation in a 15-month-old male B6,129 mouse composed of cells with small to medium round or irregular nuclei and scant cytoplasm that involve the spleen, lymph nodes, liver, kidneys, lungs, forestomach, and skin. The tumor cells express CD3 and CD8 (Fig. 10.59) and weakly CD43, but they do not express CD4, CD5 (Fig. 10.28), TDT, granzyme B, or perforin. The cell morphology and immunophenotype profile is consistent with the proliferation being a disseminated noncytotoxic CD8 T-cell lymphoma.

In conclusion, the availability of antibodies for the immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue enables the pathologist to identify marker antigens of specific cells of the hematopoietic and lymphoid systems as they relate to specific anatomic structures and the up-regulation or down-regulation of antigen expression in a cell or tissue. Finally, immunohistochemical analysis can be helpful in diagnosing and classifying reactive and neoplastic lesions and in characterizing the infiltrating cells in infectious and inflammatory processes.

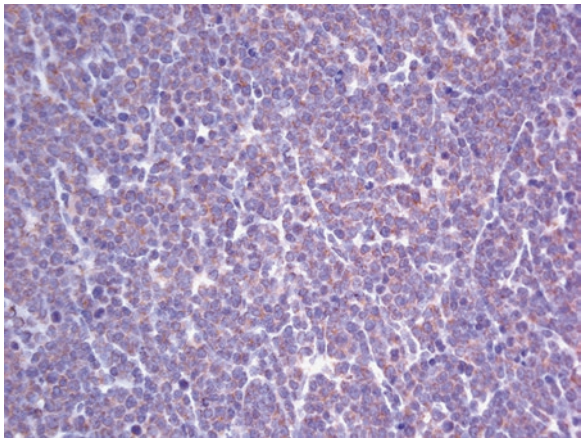

Fig. 10.57 A lymph node from a young B6,129 mouse with small blastic cells that express CD117 (KIT) on the cell membrane (Chromogen: DAB)

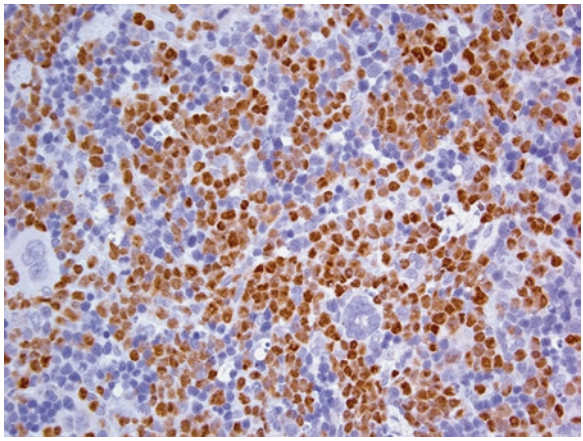

Fig. 10.58 Small blastic cells similar to those in Fig. 10.57 are present in the spleen and express nuclear PAX5, which is consistent with a pro-B lymphoblastic lymphoma (Chromogen: DAB)

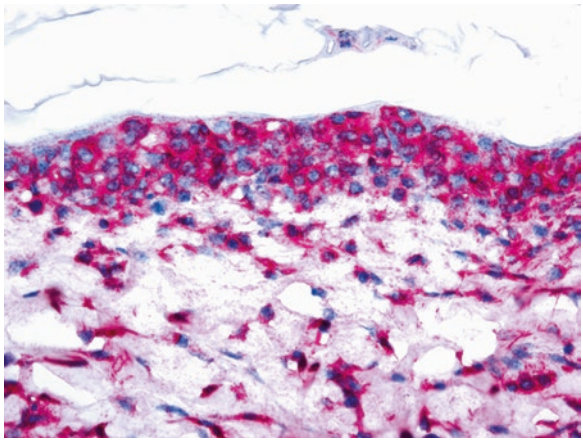

Fig. 10.59 The T-lymphoma cells infiltrating the dermis and epidermis shown in Figs. 10.27 and 10.28 also express CD8, which is consistent with a CD8 T-cell lymphoma (Chromogen: Fast Red)

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Chapter 11
Morphometry and Stereology in Immunopathology

Danielle L. Brown and Cynthia L. Swanson

Abstract In some immunopathology studies, subtle changes in particle density or quantity may be present that are below the ability of detection by routine histopathology. In those cases, quantitative analysis may be required to further categorize the change. Quantitative analysis can be performed in either a 2-dimenstional (histomorphometry) or 3-dimensional (stereology) fashion, and the type of data produced differ depending on the technique used. Morphometric analysis results in data expressed as densities or ratios, whereas stereological analysis results in absolute estimates. Both techniques have their place in immunopathology evaluation; however, the differences between them need to be understood and appreciated. This chapter outlines the principals and techniques involved in both histomorphometry and stereology and the type of data that are produced. Practical examples for using these techniques in immunopathology are also discussed.

Keywords Quantitative • Morphometry • Stereology • Image Analysis

11.1 Introduction

The pathologist has an important and unique role in the discovery and regulatory environments of drug development. However, that role is continuously changing as regulatory agencies strive for more concrete, quantitative data for decision-making in pre-clinical safety assessment. As well-trained scientists in anatomy, pathophysiology, and molecular pathways of biological systems, pathologists are called on to provide an integrated approach to these complex datasets.

Although the human eye is exceptional at pattern recognition, small differences in cell or particle density are often below its sensitivity of detection. For example, a previous study showed that even with side-by-side photomicrographs, pathologists

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could not detect a 33% reduction in hippocampal neuron number (de Groot et al. 2005). Therefore, quantitative analysis is becoming more and more requested by federal agencies for pre-clinical drug development studies.

The first step of quantitative analysis of histopathology sections is often definition of the cell or tissue type of interest using special techniques, such as histochemical stains, immunohistochemical (IHC) stains, or in situ hybridization (ISH) (see Chapters 10 and 12 for more information on the use of IHC and ISH, respectively). For the immune system, an example may be immunohistochemical staining of a particular subset of lymphocytes. This is followed by quantification of the cell type by 2-dimensional (2D) histomorphometry or 3-dimensional (3D) stereology.

Histomorphometry involves counting or measuring objects on single or multiple 2D tissue sections, either manually or through computer-assisted image analysis. The results are expressed as ratios or densities, and the data may or may not represent the tissue as a whole. On the other hand, stereology provides absolute 3D estimates of number, volume, surface area, or length through the application of rigid sectioning and sampling methods based on statistical and stochastic geometrical principles.

The following sections describe the general principles of histomorphometry and stereology and how they specifically apply to the immune system.

11.2 Histomorphometry in Immunopathology

Manual counting of cells is a slow and labor intensive process, and invariably contains observer bias. Computer-assisted quantification of digital images is more rapid and contains less observer bias. Analysis can be performed on histochemically stained, IHC stained, immunofluorescence (IF), or ISH stained tissue sections. Unlike stereology, analysis can be performed retrospectively on archived tissue. Plastic or paraffin embedded tissue as well as frozen tissue sections may be used for most purposes. Plastic or paraffin embedded tissue is preferred, as the cytologic features are better preserved when compared with frozen sections.

Whole-slide imaging systems allow analysis directly on digitally scanned slides rather than imported photomicrographs, drastically reducing time, labor and subjective bias (Fig. 11.1). The illumination is consistent across the image, an important feature when quantitating intensity or thresholding colors. An additional benefit of whole slide scanning is in the ability to view scanned slides (at various magnifications) remotely, enabling the pathologist to share the whole digital slide for further evaluation. For quantitative analysis, areas of the slide to be analyzed are either automatically selected or manually selected. Many current image analysis systems allow the use of stereology sampling principles, such as systematic uniform random sampling (SURS) in choosing fields to analyze, further reducing operator bias.

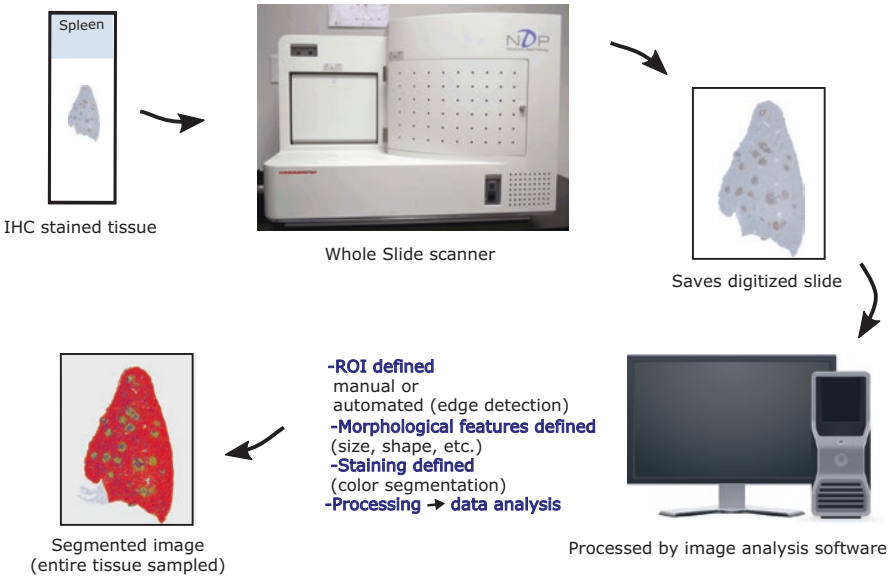

Fig. 11.1 Illustration of work-flow when using whole-slide imaging systems for morphometric analysis. Stained slides are scanned using a whole slide scanner. Digital slides are then imported into the image analysis software for whole-slide analysis

Image analysis software utilizes customized protocols for detection or quantitation of specific biomarkers or features in the tissue. Image segmentation assigns individual pixels in the digital image into categories based on color or intensity. This simplifies the image for analysis. There are a number of classification methods, the simplest being thresholding, where a certain threshold is chosen by the user to separate the pixel categories. Cluster analysis, such as K-means or Bayesian are also often used to classify pixel color and may involve "training" the software system on how to separate objects. Edge detection is useful for automatically defining the boundaries of the tissue, creating a region of interest (ROI) in which the analysis can be confined. For example, the user can train the computer to limit analysis to the cortex of the thymus and to exclude the medulla. Objects can be further classified based on size, shape and texture, and white space and artifacts can be excluded from analysis. Morphometric features such as cell (surface) area, nuclear area, cytoplasmic area, nucleus to cell area ratio, nucleus to cytoplasm ratio, cell roundness and staining intensity can be used to define classes of cells such as lymphocytes (Papakonstantinou and O'Brien 2014), particularly when lymphocyte subsets are specifically stained by IHC or other methods. An algorithm can be created that can detect nuclear, cytoplasmic, or membranous staining, which can then be quantified (Fig. 11.2). Algorithms can also be created to detect the amount of mRNA within cells or the tissue as a whole after ISH staining (Fig. 11.3).

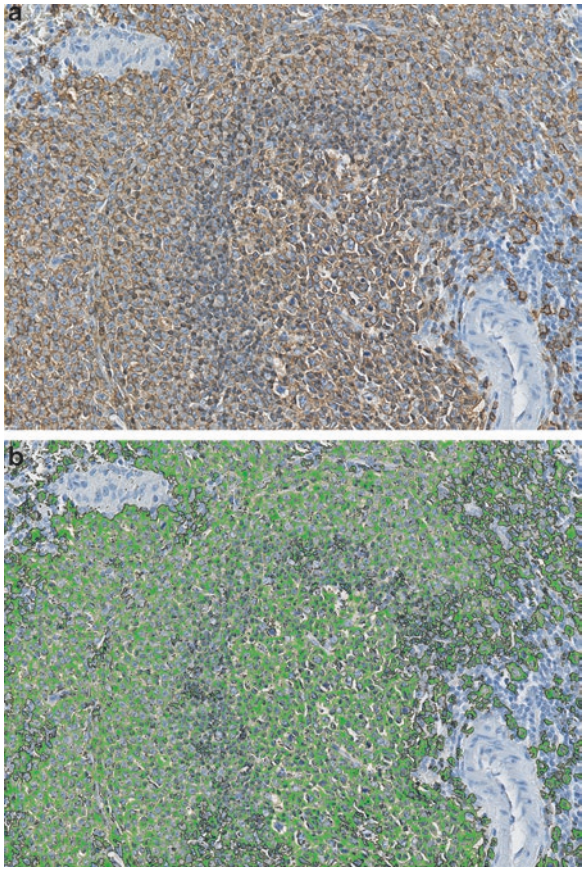

Fig. 11.2 (**a**) Photomicrograph of dog spleen stained for CD20 to identify B lymphocytes. Note the positive staining of cell membranes within the lymphoid follicle (*brown*). (**b**) Application of an image analysis algorithm to identify positive staining (*green label*). IHC for CD20, DAB with hematoxylin counterstain, 20× magnification

Quantifying cell proliferation and cell death can also be useful to the pathologist in both a diagnostic and experimental setting. IHC stains for cell proliferation include Ki67, bromodeoxyuridine (BrdU), and proliferating cell nuclear antigen (PCNA). Algorithms can be created to identify and count positively and negatively stained nuclei in order to calculate a labeling index (LI), which is the percentage of positively stained cells (Marinaccio et al. 2015; Chabot-Richards et al. 2011) (Fig. 11.4). Similarly, algorithms can be created to count positive caspase-3 or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)-labeled cells through identification of characteristic condensation/decrease in cell size as well as positive staining to distinguish cells undergoing apoptosis and calculate an apoptotic LI (Krajewska et al. 2009; Garrity et al. 2003).

Automated image capture and analysis can also be used for high-throughput screening of tissues using certain biomarkers. Tissue microarrays consist of many tissue cores on a single slide, which are then stained via IHC or ISH. Image analysis protocols can then be created to analyze and record data for each core sample. This

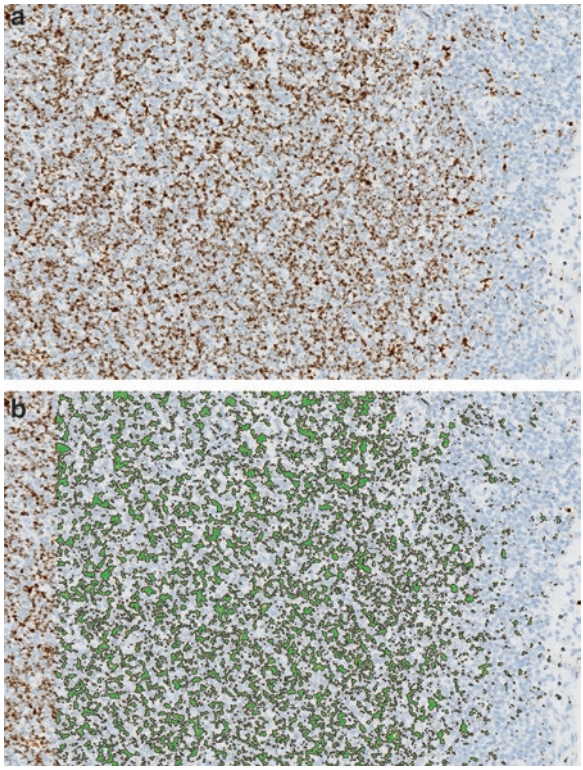

Fig. 11.3 (**a**) Photomicrograph of rat lymph node stained for CD4+ T cells by in situ hybridization (ISH). Note the variably-sized brown-stained "dots" within the cells representing transcripts of CD4 mRNA. (**b**) Application of an image analysis algorithm to identify positive staining within the cells (*green label*). ISH for CD4, DAB with hematoxylin counterstain, 20× magnification

allows for efficient, rapid quantitation of multiple tissues and biomarkers under standardized conditions (Jawhar 2009; van Zwieten 2013).

Antibodies to numerous biomarkers are available to characterize cells involved in inflammation as well as lymphocyte populations through IHC staining (Sconocchia et al. 2014). However, many of the hematolymphoid antigen markers are cell lineage-associated, but not cell lineage-specific, and a panel of antibodies may be required to confirm cell lineage (Rehg et al. 2012). Similarly, a panel of mRNA probes can be used to detect these cell markers at the RNA level using ISH. In both cases, staining can be performed for two or more markers on the same slide, and image analysis can be utilized to detect co-localization of these markers within cells. The number of single positive, double positive, and negative cells can then be determined. This may be especially useful for determining lymphocyte lineage through the use of cell markers such as CD3, CD4, and CD8.

In addition to automated image analysis, manual histomorphometry on digitally scanned slides may also be of use in immunopathology. Linear measurement of the thickness of the thymic cortex or marginal zones in the spleen are some examples (Fig. 11.5).

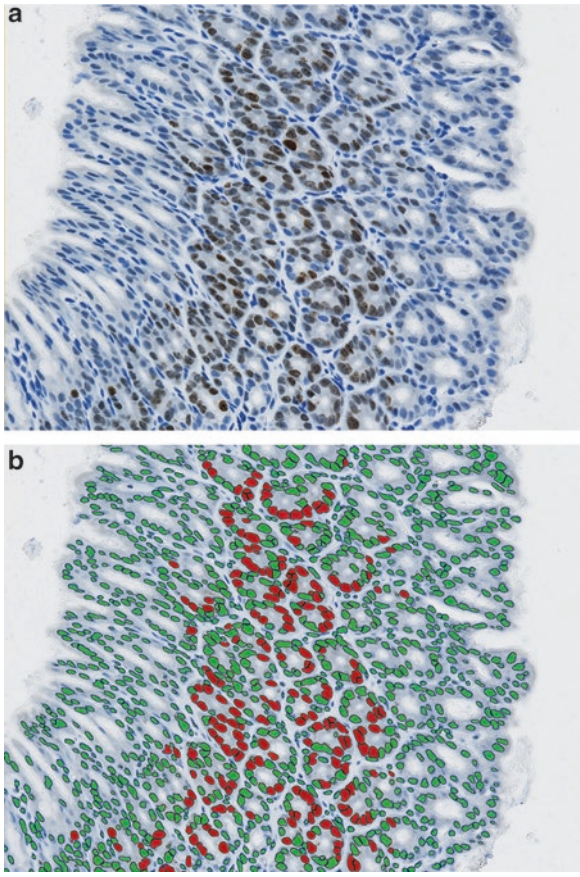

Fig. 11.4 (**a**) Photomicrograph of rat stomach stained for Ki67 by immunohistochemistry (IHC). Note the positively stained nuclei (*brown*) and the negatively stained nuclei (*blue*). (**b**) Application of an image analysis algorithm to identify positively stained cells (*red label*) and negatively stained cells (*green label*). These cells can then be counted by the software to calculate a labeling index. IHC for Ki67, DAB with hematoxylin counterstain, 20× magnification

11.3 Limitations of Histomorphometry

Although histomorphometry is often a nice screening tool for investigative studies to detect quantitative changes and can be useful in gleaning information about the tissue sections that are being evaluated, the results are biased (or inaccurate) due to several assumptions placed on the tissue sections. For example, histomorphometry assumes that the tissue is homogeneous throughout. Analysis is performed on single (or sometimes multiple) 2D tissue sections, without regard for the tissue structure as a whole and without a universal sampling paradigm. This leads to data that may not necessarily represent the tissue being sampled. For example, if a particular lymphocyte subset is counted on a single section through the thymus, where the tissue was sectioned (i.e. the amount of cortical tissue versus medullary tissue present) will greatly affect the outcome.

Histomorphometry also assumes that there is no change in organ size during tissue processing. We know that is not the case, particularly with paraffin processing. One study found that glomerular volume estimates were 40% lower for kidneys

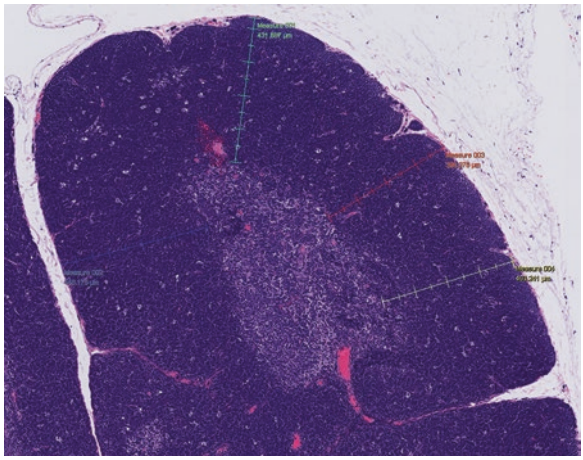

Fig. 11.5 Photomicrograph of rat thymus with linear measurements to evaluate the thickness of the thymic cortex. Hematoxylin and eosin (H&E) stain, 5× magnification

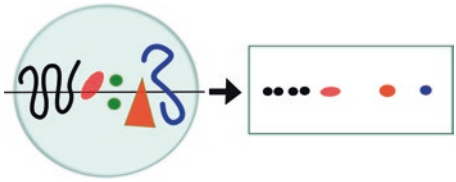

Fig. 11.6 Illustration of what occurs when a 2-dimensional (2D) section is taken through a 3-dimensional (3D) structure, as the case when tissues are sectioned for microscopic evaluation. The number of objects in 2D does not match that in 3D, particularly for objects that move in and out of the plane (*black object)*. Additionally, all information on size, shape, and orientation is lost, and some objects are not captured in the section at all (*green objects*)

embedded in paraffin when compared with those embedded in methacrylate (Miller and Meyer 1990). It is known that density estimates, such as those obtained through 2D histomorphometry, are especially sensitive to the effects of tissue shrinkage, leading to overestimation of cell number and underestimation of cell volume. Furthermore, tissue from control and test article-treated animals may respond differently to this shrinkage. For example, the testes of rats and hamsters treated with testosterone and estradiol were shown to experience a higher degree of tissue shrinkage due to processing than testes of untreated animals (Mendis-Handagama and Ewing 1990; Mendis-Handagama 1992).

One of the largest limitations of 2D histomorphometry is the loss of information about particle size and shape when a 3D tissue is reduced to a 2D histological section, leading to a loss in mathematical relationship between the number of 2D profiles and the number of 3D particles (Fig. 11.6). The presence of profiles on a 2D section is influenced by the size, shape, orientation, and distribution of the particles within the tissue. For example, larger particles and/or those oriented perpendicular to the sectioning plane are more likely to be counted. This results in an overestimation of particles in general, particularly those that are larger or have this particular orientation. Several studies have illustrated this disagreement between 2D profile

counting and actual cell number, including studies by Pakkenberg et al. (1991), in which 2D profile counting within the substantia nigra was demonstrated to give a 30–40% overestimation of neuron number. Other studies have shown even more dramatic differences, some even showing a result that was the opposite direction from the truth. For example, a study estimating trabecular bone number in minipigs demonstrated a reduced number in treated pigs using 2D estimation whereas 3D stereological methods showed an increased number (Boyce et al. 1995).

All of the above limitations are examples of bias. Biased data are inherently inaccurate and may not represent the true population mean, even if the data appear to be reproducible. Because of the biases of 2D histomorphometry, several professional and regulatory societies have published best practice manuscripts on quantitative assessment, recommending the use of stereology in place of histomorphometry. This includes the American Thoracic Society and European Respiratory Society (Hsia et al. 2010), the American Society of Nephrology (Madsen 1999), and the editors of the Journal of Comparative Neurology (Saper 1996).

11.4 Stereology in Immunopathology

Stereology is a technique for estimation of absolute number, volume, surface area, or length of objects based on statistical principles and stochastic geometry. Unlike 2D histomorphometry, it does not make assumptions about the tissue or objects within the tissue; it is design-based rather than assumption-based. Therefore, it is unbiased, yielding data that are statistically closer to the true population mean. These data can either be closely clustered together and precise (reproducible), or more highly variable and imprecise. In stereology, precision can be estimated and improved upon through more rigorous sampling (explained in additional detail below). By contrast, in histomorphometry, there is no way to measure precision and therefore, it cannot be improved.

Recent technological advances, particularly the use of computer-aided analysis of digital slides, have made stereology both more approachable as well as more efficient. One of the most advantageous aspects of stereology is that the same principles can be applied to virtually any tissue, including those of the immune system.

11.4.1 Tissue Sampling for Stereology

One of the first and foremost steps of any stereology study is accurate sampling of the tissue or region of interest. Sampling of tissues for stereology follows a procedure known as systematic uniform random sampling (SURS), which ensures that every structure of interest within the tissue has an equal chance of being sampled. The length (or widest point) of the tissue or region of interest is measured and a

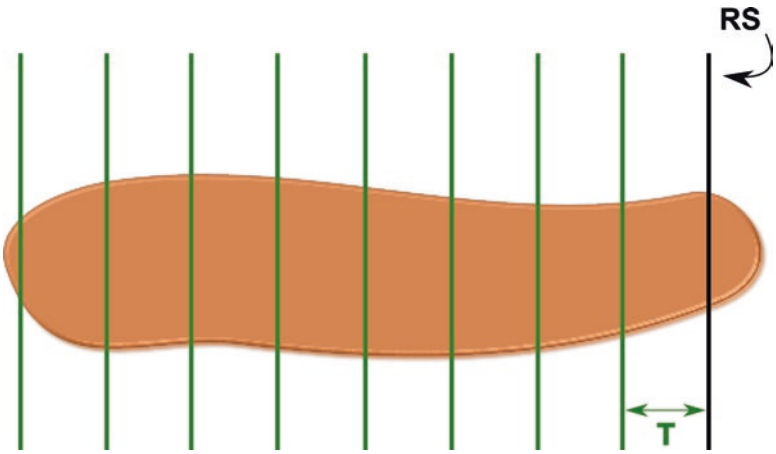

Fig. 11.7 Sectioning of a tissue by systematic uniform random sampling (SURS). The length of the tissue is measured and a sampling interval (*T*) is chosen that will result in 8–10 sections through the tissue. A random start (*RS*) is chosen between 0 and T for the first section. Subsequent sections are captured at regular intervals of *T* across the tissue

sampling interval (T) is chosen that will capture approximately 8–10 sections through the tissue. The first sampling interval is chosen at random (random start) by selecting a random number between 0 and T. Sections are then obtained at regular intervals of T until the tissue has been exhaustively sampled (Fig. 11.7). For small tissues such as lymph nodes, this can be accomplished entirely at the microtomy stage. For example, the lymph node is processed whole in paraffin, and the widest part of the tissue is measured post-processing using calipers. The sample is then embedded whole in paraffin. The width is divided by 10 to obtain T, and a random start between 0 and T is chosen for the first section. An automated microtome is used that has been calibrated for the section thickness (Fig. 11.8), so that the microtome can zeroed after the first section is taken and the tissue is microtomed until the next sampling interval is reached. Sections are taken at each sampling interval until the tissue block is exhausted. It is recommended to take several additional sections at each sampling interval that can be used for routine histopathology, additional special stains, or as backups if needed.

For medium-sized tissues such as rat spleen, the tissue can be cut into slabs using SURS prior to the final embedding stage (Gundersen et al. 2013). If paraffin embedding will be used, cutting the tissue into slabs must be performed subsequent to tissue processing; otherwise tissue deformation during processing will affect the sampling intervals. The entire tissue can be processed in paraffin, the length measured post-processing, and the entire tissue embedded in a paraffin mold. If a mold of the correct size is not available, the authors have constructed large molds out of aluminum foil or aluminum baking pans when necessary (Fig. 11.9). The length is divided by 8–10 to obtain T, and a random start is chosen between 0 and T. Using a mini hacksaw and a cutting guide (such as a miter box), the large paraffin block is sawed into slabs at

Fig. 11.8 An automated microtome is used for exhaustively sectioning through tissue blocks using systematic uniform random sampling (SURS) for stereology. After sections are collected at each sampling interval, the read-out on the microtome can be zeroed (*arrow)* and the block can be further sectioned until the next sectioning interval has been reached

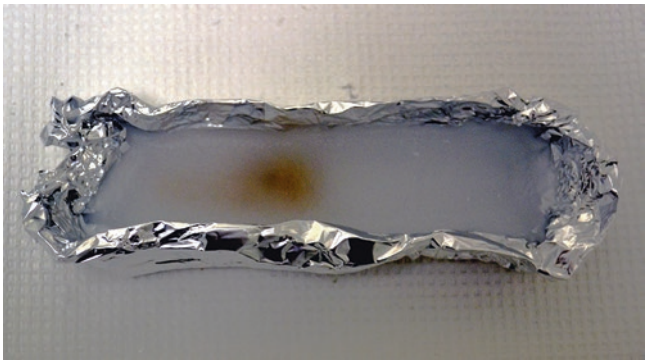

Fig. 11.9 Creation of a make-shift paraffin mold using aluminum foil for SURS sectioning of the entire paraffin-embedded tissue

regular intervals of T with a goal of obtaining 8–10 slabs (Fig. 11.10). That may not be possible with tissues that are still relatively small, such as rat spleen or thymus. In those cases, fewer slabs may be obtained. Slabs can then be re-embedded using typical embedding molds. Sections will be microtomed from the first clean tissue face (free from sawing artifacts) for each slab. A pilot study can be helpful to determine

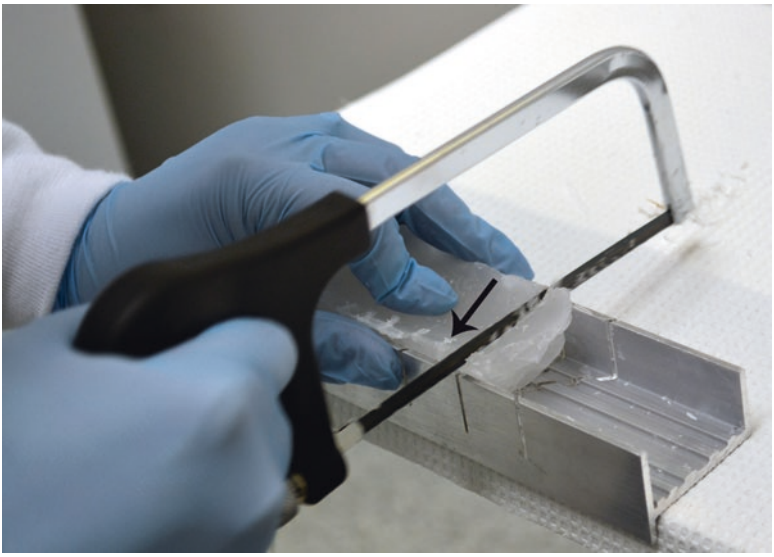

Fig. 11.10 SURS sectioning of a tissue fully embedded in paraffin using a miter box and a mini hacksaw. Note the notches made on the block prior to sectioning (*arrow*). These identify the sectioning interval (T) as well as the random start to guide the sectioning

the distance needed to obtain a clean tissue face, and that distance can be applied to all study animals and blocks to maintain a uniform sampling interval.

If the tissue will be embedded into plastic (glycol or methyl methacrylate), medium-sized tissues can be embedded into agar and sectioned into slabs using a tissue slicer (Fig. 11.11). The slabs are then processed and embedded in plastic. The shrinkage and tissue deformation observed with plastic embedding is much less than that observed with paraffin; therefore, it is the recommended embedding medium if volume estimates are to be obtained. However, IHC staining on plastic sections can be extremely difficult. In those cases where IHC staining is needed, volume can be estimated on paraffin embedded tissue by correcting for shrinkage (Gundersen et al. 2013). The tissue is weighed both prior to and following paraffin processing and 3D global shrinkage is estimated using the following equation: $1 - W_{fix}/W_{par}$, where W_{fix} is the pre-processing weight and W_{par} is the post-processing weight. The final volume estimates are corrected for shrinkage using the following equation: V(corrected) = V/(1 − 3D global shrinkage). Certain stereological methods such as the fractionator for cell or object number are insensitive to shrinkage (Gundersen 1986).

For large tissues such as canine or non-human primate spleen, the tissue can be sampled prior to processing using cylindrical knives and a cutting guide, a method known as the “fast Fractionator”. This method is outlined in detail in Gundersen et al. (2013).

Another consideration for tissue sampling is whether or not the sections need to be isotropic uniform random (IUR). Isotropy means that all directions have an equal

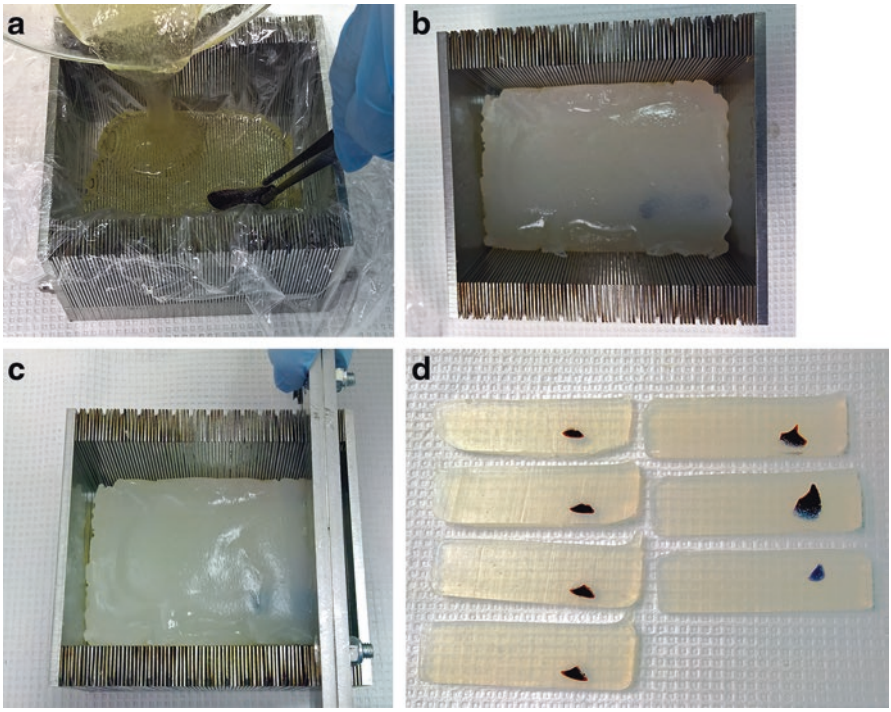

Fig. 11.11 Embedding a rat spleen in agar and sectioning by SURS using a tissue slicer. (**a**) Plastic wrap is placed within the tissue slicer to use it as a mold. The tissue is placed within the slicer and hot (approximately 50 °C) agar is poured into the mold. (**b**) The agar is allowed to harden prior to sectioning and the plastic wrap is removed. (**c**) Special knives are used to section the tissue by SURS, using a random start and regular sampling interval. Each slot within the tissue slicer represents 1 mm. (**d**) Tissue slabs are placed in order, same side up. Excess agar is removed from around the tissue and the tissue is further processed and embedded in glycol methacrylate (not shown)

chance of being chosen, and is important for estimation of surface area, length, and cell volume (but not number or tissue volume) of objects that have anisotropic (non-random) orientation. This includes objects such as capillaries or nerve fibers. For larger tissues, a common technique for isotropic sectioning is the orientator and for smaller tissues, a common technique is the isector (Nyengaard and Gundersen 1992). Detailed descriptions of these techniques are given in Nyengaard and Gundersen (2006). In cylindrical or spherical tissues with a vertical axis, such as lymph nodes, vertical sections can be taken in lieu of isotropic sections (Baddeley et al. 1986). These are captured by randomly rotating the tissue around its vertical/longitudinal axis and then taking tissue sections parallel to this axis.

Additional sampling techniques applicable to other organ types are detailed in Gundersen et al. (2013). Regardless of the sampling methodology applied, the general goal in stereology is to obtain approximately 8–10 sections through the tissue or region of interest, either in a single step or several sub-sampling steps.

11.4.2 Sampling Fields of View and Applying Stereological Probes

After the tissues have been sampled, microscopic fields of view are captured on the resulting histologic sections, most often via a computerized system. The fields of view can be captured using scanned virtual slides, or via a live microscope equipped with a camera and motorized stage (Fig. 11.12). The most common method for capturing microscopic fields of view is the fractionator, in which a certain percentage of the tissue is sampled (defined by the user), known as the areal sampling fraction (asf). Fractionator sampling applies SURS principles to the tissue sections in that the first field of view is chosen at random and subsequent fields are chosen at regular intervals necessary to obtain the desired percentage of sampling (Fig. 11.13a). A more efficient method for capturing microscopic fields of view is through Proportionator™ sampling, which uses non-random sampling that is guided by automated image analysis (Gardi et al. 2008a; 2008b). The user creates an image analysis algorithm to detect the feature of interest within the section (often by positive IHC staining) and the software assigns a "weight" to each microscopic field of view that corresponds to the probability of having a positive count in that field. The computer then uses these probability and "weight" estimates to drive the sampling so that fields with a higher probability of having a positive count are more likely to be chosen (Fig. 11.13b). The end goal in most stereology studies is obtaining between 100 and 200 positive counts per animal, which is accomplished more efficiently with Proportionator sampling in many cases.

Stereologic "probes" or test systems are then applied to chosen fields of view and interactions between the objects of interest and the test system are counted. The type

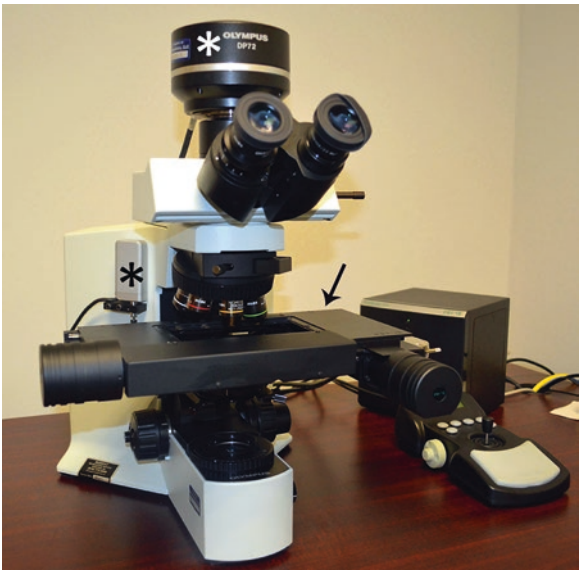

Fig. 11.12 Live microscope setup for stereology. The microscope contains a camera (white a*sterisk*), motorized stage (*arrow)* and length gauge (*black asterisk*)

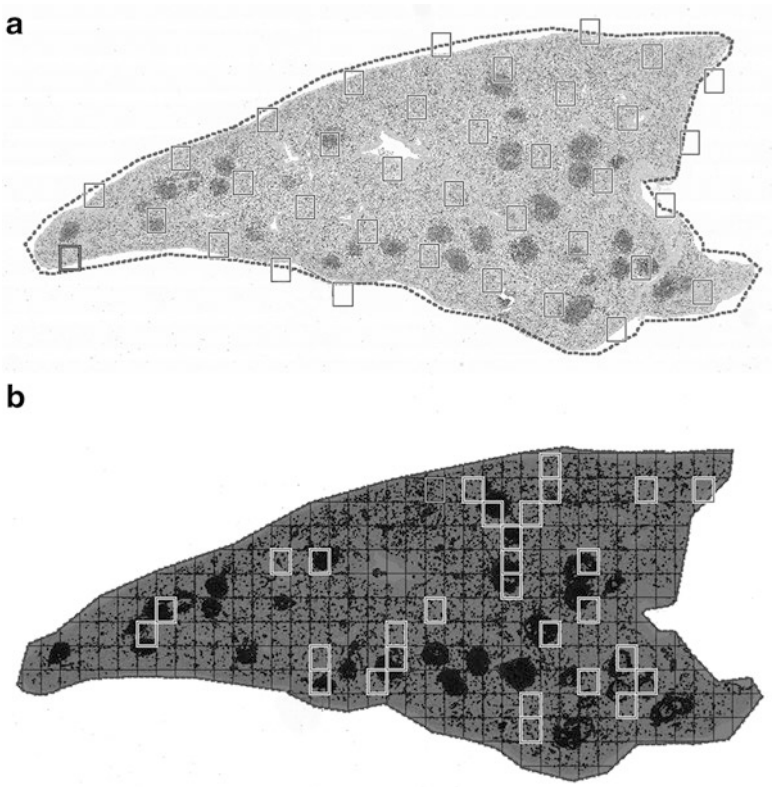

Fig. 11.13 Sampling of microscopic fields for stereology on dog spleen stained for CD20 by IHC. (**a**) Fractionator sampling, which uses SURS to choose microscopic fields of view. Note that the chosen fields of view (*boxes*) are regularly spaced across the tissue. (**b**) Proportionator sampling, which utilizes image analysis to guide sampling. The chosen algorithm is applied to the image and chosen fields of view (*boxes*) are centered over regions with increased CD20 positivity (*dark areas*)

of test system(s) chosen depends on the estimate of interest (number, volume, surface area, or length). The sum of dimensions of the probe and the estimate of interest is always 3 (Table 11.1). Therefore, if volume is of interest, which is 3-dimensional, a test system with no dimensions is added to the fields of view (i.e. points). Similarly, if surface area (2-dimensional) is of interest, 1-dimensional lines are added to the fields of view and if length (1-dimensional) is of interest, a 2-dimensional plane is sampled. If number is the desired estimate, a 3-dimensional test system is used, also known as the disector. Unlike the other test systems, the disector is applied at the time of tissue sectioning. There are two types of disectors that can be used; physical disectors are consecutive thin (3–5 μm) sections, whereas optical disectors are single thick (30 μm or more) sections that are captured at each sampling interval. The disector ensures that objects are sampled according to their number rather than their size, shape, or orientation.

Table 11.1 Geometrical relationship between structural feature of interest and stereological test system

Structural feature of interest (dimension)	Stereological test system (dimension)	Counting event
Volume (3)	Point (0)	Point "hits" object
Surface area (2)	Line (1)	Line "intersects" object
Length (1)	Plane (2)	Profile present within plane
Number (0)	Disector (3)	Unique counting feature present within counting frame in disector

Application of a stereological test system to fields of view is illustrated in Fig. 11.14. For volume, the intersections between the points and the tissue or other object of interest are counted (Fig. 11.14a). Similarly, for surface area, the intersections between the lines and the objects of interest are counted (Fig. 11.14b). For length and number, an unbiased counting frame is applied to each field of view. The unbiased counting frame contains two "inclusion" lines and two "exclusion" lines. Objects are counted if they are within the counting frame or touching the inclusion lines, but not if they are touching the exclusion lines (Fig. 11.14c). This prevents an object from being counted twice if side-by-side microscopic fields of view are evaluated. If length is the desired estimate, the number of objects within the counting frame are counted on each field of view without the use of the disector. For number using the physical disector, the computer captures matching fields of view from both consecutive sections and places them side by side on the computer screen. A unique counting feature is chosen (i.e. nucleus or nucleolus), and the cell is counted if that unique counting feature is present on one side of the disector but absent on the other (Fig. 11.15). If the optical disector is used, the computer chooses the microscopic fields of view using a motorized stage and the user slowly focuses down through the section on high magnification oil objective, counting the cells as the unique counting feature comes into crisp focus. An important aspect that needs to be considered when using optical disectors is the possibility of artifacts or loss of cells at the top and bottom of the section; because of that issue, "guard zones" need to be established in which cells are not counted (Fig. 11.16).

11.4.3 Applications of Stereology in Immunopathology

The most common applications of stereology to the immune system are volume and number. Volume estimates may involve the entire tissue, such as the volume of the whole thymus to detect treatment-related thymic atrophy, or the volume of tissue compartments, such as the thymic cortex, germinal centers within the lymph nodes, or marginal zones in the spleen. Number estimates may involve total number of a certain subset of lymphocytes (e.g. CD4+ T lymphocytes) within a tissue when stained with an immunohistochemical marker or in situ hybridization probe.

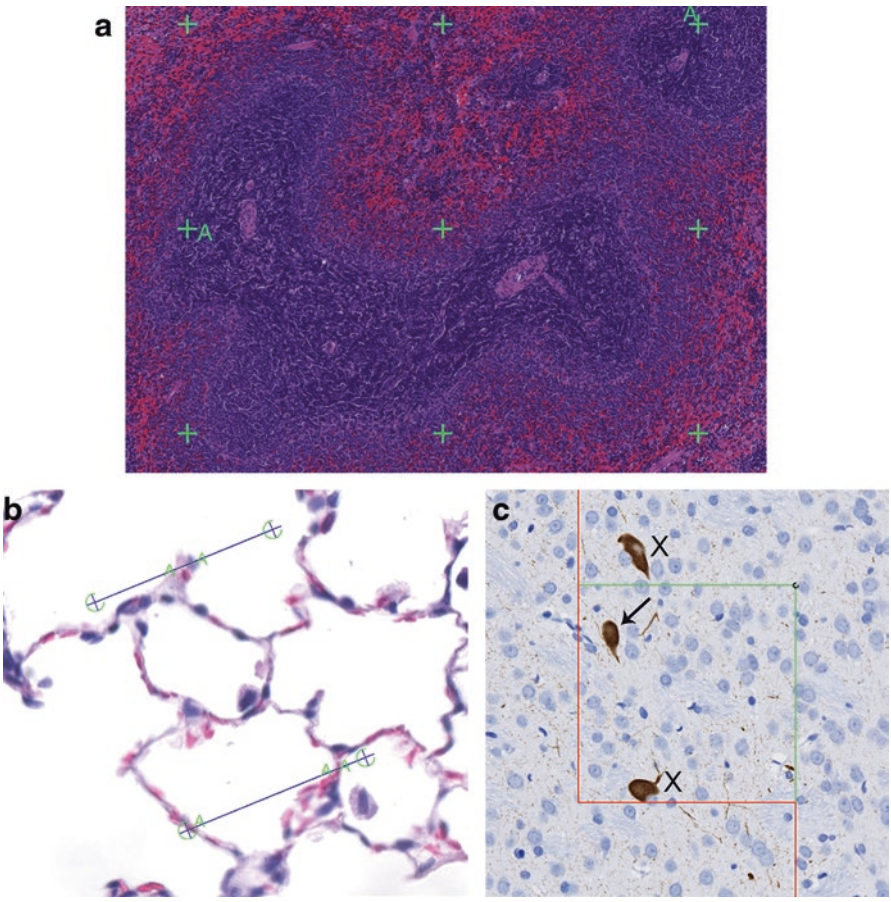

Fig. 11.14 Application of stereologic test systems to sampled microscopic fields of view. (**a**) Volume estimation of splenic white pulp. A point probe is applied to the field of view (*green crosses*) and interactions between the points and the white pulp (*dark purple areas*) are tagged (*A*). H&E stain, 5× magnification. (**b**) Estimation of alveolar surface area in the lung. A line probe is applied to the field of view (*blue lines*) and intersections between the alveolar tissue and the lines are tagged (*A*). H&E stain, 40× magnification. (**c**) Estimation of neuron number in the brain. An unbiased counting frame is applied to the field of view and contains inclusion lines (*green lines*) and exclusion lines (*red lines*). Cells are counted if they are within the counting frame (*arrow*) but not if they are outside of the counting frame or touching the exclusion lines (*X*), IHC for choline acetyltransferase (ChAT), DAB with hematoxylin counterstain, 40× magnification

To estimate total tissue volume, the Cavalieri estimator is used. The entire tissue is sectioned by SURS to obtain eight to ten sections through the tissue. Point probes are then overlaid on the tissue sections, either at the subgross or low magnification microscopic level, and the number of points hitting the tissue are counted (Fig. 11.17). The total volume is then estimated by the Eq. $V = \Sigma P \times a(p) \times T$; where ΣP is the sum of the points hitting the tissue across all tissue sections, $a(p)$ is the cross-sectional area assigned to each point (Fig. 11.18), and T is the distance between tissue sections.

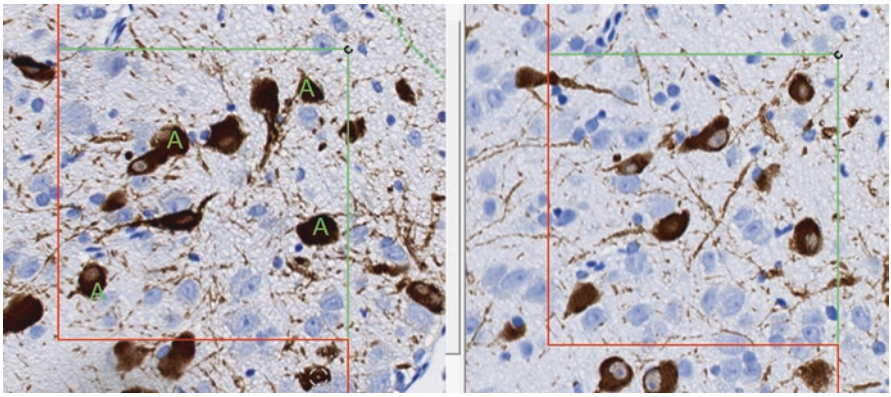

Fig. 11.15 Counting cells within a physical disector. Matching microscopic fields are displayed by the software and a unique counting feature is chosen (in this case, the nucleus). Cells are counted if they are within the unbiased counting frame and the unique counting feature is present in one field but not the other (*A*). IHC for ChAT, DAB with hematoxylin counterstain, 40× magnification

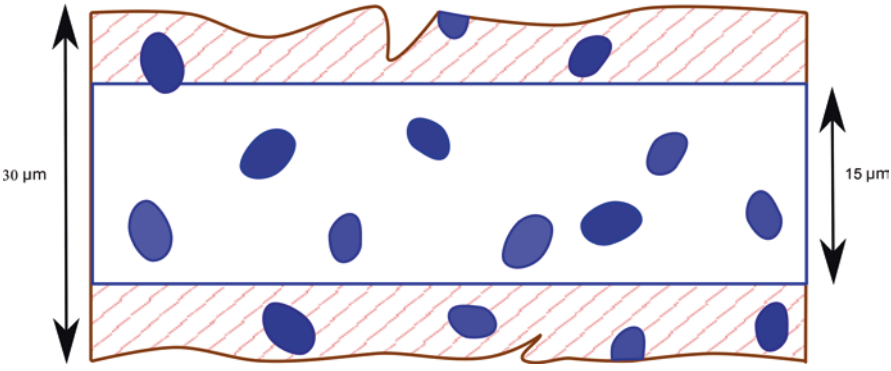

Fig. 11.16 Illustration of an optical disector as seen from the side. The disector is approximately 30 µm thick; however, there is some non-uniform shrinkage in section thickness present. There are artifacts (knife cuts) on the top and bottom of the section, and some cells (*blue*) are not wholly present along the top and bottom edges. Because of these issues, guard zones (*red lines*) need to be utilized, which are regions at the top and bottom of the section in which cells are not counted. The size of the guard zones are determined in a pilot study. It is important that the height of the disector (area in which cells are counted) be at least 15 µm

To estimate tissue compartments, the volume fraction is first estimated. After SURS sampling of the tissue, microscopic fields of an appropriate magnification are captured, often also by SURS. Double-point probes are overlaid on the chosen microscopic fields and are comprised of one set of points that are closer together and used to evaluate the feature of interest (e.g. the marginal zones of the spleen), and another set that are farther apart and used to evaluate the reference space (i.e. the entire spleen). Often the points used to evaluate the total tissue are encircled

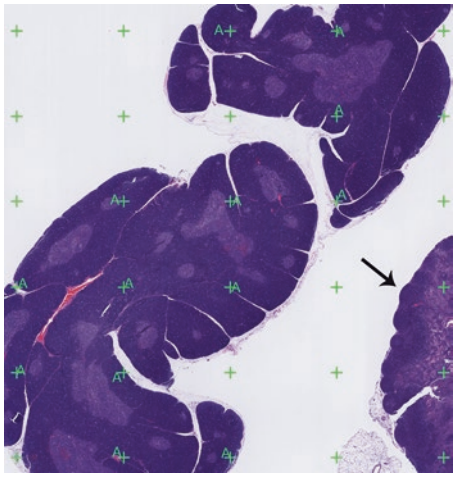

Fig. 11.17 Estimation of total tissue volume of rat thymus. A point probe (*green crosses*) is randomly placed over the tissue and the interactions between the points and the thymus are tagged (*A*). Note that the points overlying the lymph node (*arrow*) are not counted. H&E stain, 1× magnification

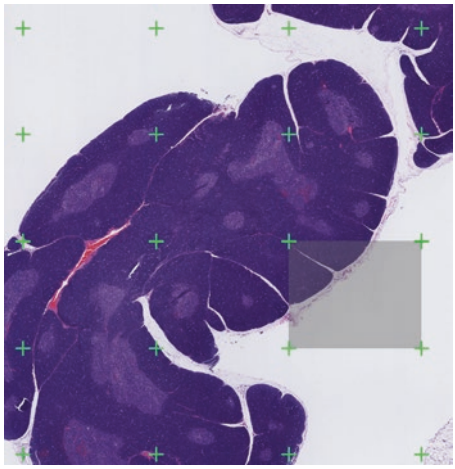

Fig. 11.18 Same image of rat thymus from Fig. 11.17, with illustration of the area per point (*shaded box*). H&E stain, 1× magnification

whereas the others are not (Fig. 11.19). Interactions between the points and the tissue or subregion are counted. The volume fraction is then estimated by the Eq. $V_V = [\Sigma P_i \times A(p_i)] / [\Sigma P_r \times A(p_r)]$; where ΣP_i is the sum of the points hitting the tissue subregion of interest (i.e. splenic marginal zones), $A(p_i)$ is the area per point for the set of points evaluating the subregion; ΣP_r is the number of points hitting the reference space (i.e. the spleen), and $A(p_r)$ is the area per point for the set of points evaluating the reference space. Once the volume fraction is determined, it is multiplied by the total tissue volume (determined by the Cavalieri estimator as described above) to determine the absolute volume of the tissue compartment, as in the equation $V_{subregion} = V_v \times V_{tissue}$.

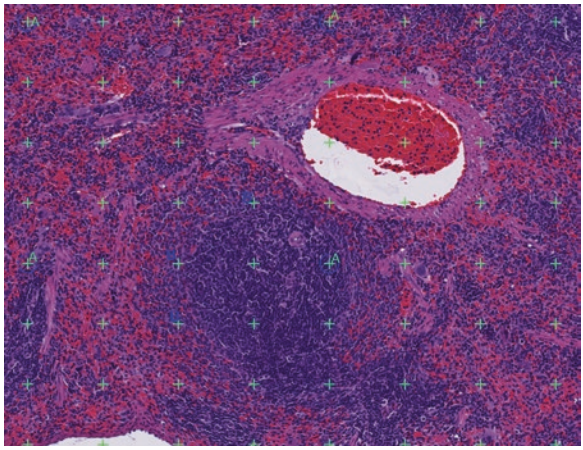

Fig. 11.19 Estimation of volume fraction of splenic marginal zones. A point probe (*green crosses*) is randomly placed over the field of view and the interactions between the points and the splenic marginal zones are tagged (*B*). In addition, the interactions between the encircled points and the spleen as a whole (reference space) are tagged (*A*). H&E stain, 10× magnification

Cell number is also a common desired endpoint for immunopathology studies. This can be accomplished through SURS sampling of the tissue to obtain physical or optical disectors, and then application of Fractionator or Proportionator sampling to capture microscopic fields of view, as described above. The user then tags all cells that are within the unbiased counting frame as they are in focus (optical disector) or when a unique counting feature is on one of the matching high magnification fields and not the other (physical disector), as described above. The sum of all of the counts for Fractionator, or all of the weighted counts for Proportionator (calculated as the raw count multiplied by the inverse of the probability of having a positive count for each sampled field of view) are then used in an equation for absolute number. For physical disectors, the equation used is $N = \Sigma Q \times 1/\text{bsf} \times 1/\text{ssf} \times 1/\text{asf}$; where ΣQ is the sum of the counts or weighted counts (divided by 2 if counting is performed in both directions of the disector), bsf is the block sampling fraction (i.e. 1/3 if every third block of the tissue is used for analysis), ssf is the section sampling fraction (section thickness divided by distance between sections), and asf is the areal sampling fraction (percentage of the tissue sampled, only used for Fractionator sampling). For optical disectors, the height sampling fraction (hsf) also needs to be taken into account, which is the height of the disector (the thickness in which cells are counted once the guard zones are excluded, i.e. 15 μm) divided by the average section thickness. At each microscopic field, the section thickness is measured by a length gauge attached to the microscope (see Fig. 11.12). This is logged by the computer and averaged over all fields of view (often weighted for the number of counts per field). The equation used for absolute number in optical disectors is thus $N = \Sigma Q \times 1/\text{bsf} \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{hsf}$.

Other stereological estimates that would be less commonly utilized for immunopathology include surface area, length, and cell volume. Procedures for these estimates can be found in detail in other publications (Baddeley et al. 1986;

Gundersen 1988; Jensen and Gundersen 1993; Mattfeldt et al. 1990; Gundersen et al. 2013; Howard and Reed 2005). An important thing to note is that preparation of tissue sections for estimation of surface area, length, or cell volume requires additional steps to ensure isotropy, or that all directions have an equal chance of being sampled (Mattfeldt et al. 1990; Gundersen et al. 2013). Alternatively, vertical sections can be used in which the vertical (often longitudinal) axis of the tissue is kept track of throughout the sampling process (Baddeley et al. 1986; Gundersen et al. 2013). Methods for capturing isotropic or vertical sections are outlined above.

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Chapter 12
Molecular Techniques in Immunopathology

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Abstract Molecular pathology is a rapidly progressing field. As members of a multidisciplinary investigational team, pathologists are increasingly being asked to have a basic understanding of molecular techniques, their usefulness, and their limitations. The correlation of the morphologic phenotype with the cellular and gene based pathogenesis of disease lies within this realm. This chapter will focus on appropriate sample collection for molecular pathology as well as the utility of various molecular techniques as tools from for use in drug discovery and through development.

Keywords In situ hybridization (ISH) • Sample preparation • PCR analysis (Polymerase Chain Reaction) • Next generation sequencing • Formalin fixed paraffin embedded (FFPE) tissue blocks

12.1 Introduction

The term "molecular pathology" can mean different things to different people. The commonality of most definitions may be described as the correlation of morphologic phenotype with cellular and gene based pathogenesis of disease. Pathologists provide expert opinions for evaluation and interpretation of tissue morphology and now are being challenged to integrate molecular data. Hematoxylin and eosin tissue staining, immunohistochemistry (IHC) and in situ hybridization (ISH) are mainstays for the discipline with the use of digital image analysis for quantitative assessments. Additionally, molecular pathology can

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include cross disciplinary input from biochemistry, proteomics, genomics, genetics, bioinformatics, systems biology, in vivo molecular imaging, and other sciences. Many of these disciplines have traditionally focused on samples other than tissue, notably blood, urine and cell culture, but they are now increasingly being applied to tissue.

Working in teams becomes a necessity as no one person can master the necessary skill sets required to generate fully integrative, translational and relevant results. Pathologists can and should function as gatekeepers to insure appropriate tissue quality, characterization and sampling as well as to provide integrated, holistic perspective for data interpretation.

Examples of molecular applications to discovery, preclinical and clinical investigations of the immune system will be highlighted. In the clinical arena, molecular diagnostics have become increasingly essential for oncology, specifically hematologic neoplasia and lymphomas. Human molecular oncology laboratories offer a wide range of PCR, cytogenetic and genomic assays for tumor classification. Similarly, companion diagnostics seek to identify subgroups of tumors susceptible or resistant to specific therapeutic regimens, most notably IHC for human breast cancer. Perspective and knowledge is required to adapt and apply these tools to non-human species. As an example, human genes for T cell receptors and immunoglobulins can be probed for rearrangements to assess clonality of proliferating lymphoid tissue, but species differences in gene rearrangement mechanisms limit universal application. Distribution and expression of receptors and signaling proteins on immune cell populations also varies greatly by species. Translation from human to relevant animal models and vice versa resides in the realm of the pathologist.

Investigation of infectious and inflammatory disease using both human tissues and animal models increasingly utilizes genomics, sequencing, epigenetics, proteomics, bioinformatics, and systems biology. Diagnosis of infectious disease that may be seen as opportunistic infection in immunocompromised hosts extensively relies on PCR as well as traditional approaches. The mere presence of an infectious agent does not equate with disease. The pathologist is positioned to play a key role in holistic interpretation of diverse data, but must be adequately versed in the disciplines to evaluate data quality, technical limitations and strength of data. Confirmation of the appropriate morphologic change, application of molecular data to the disease condition and localization within the diseased tissue has great relevance for understanding and implementing therapeutic intervention.

Previous chapters have focused on immunohistochemistry, flow cytometry, and stereology. This chapter will primarily focus on preparation of samples for molecular analysis, Polymerase Chain Reaction (PCR) and In Situ Hybridization (ISH).

12.2 Sample Preparation for Molecular Analysis

Sample quality is one of the most important aspects to consider when including molecular techniques in a study. When designing a study, sample procurement, fixation, processing, embedding, and storage should be clearly defined as they are all critical to the outcome of these evaluations. When performing a retrospective analysis, it is important to understand that variability in the above procedures can lead to nucleic acid degradation and can affect the outcome.

Histopathology remains the cornerstone of pathologic assessment. Formalin fixed paraffin embedded (FFPE) tissue blocks are still the main endpoint evaluated from a pathology study. FFPE are widely available in archives and are often associated with a wealth of metadata (including stage of disease and response to therapy) which can be used in retrospective analysis.

The process of creating and storing FFPE blocks preserves the tissue architecture, but it results in damage to nucleic acid content. Nucleic acids (especially RNA) are sensitive to the presence of endogenous nucleases present in tissue or cells freshly harvested from their native environment. Nucleic acids are also sensitive to any exogenous nucleases that may be introduced during sample handling or that may be present as contamination on instruments used during tissue harvest.

12.2.1 Procurement and Fixation

The time elapsed between removal of a tissue from its nutrient supply and its immersion in a fixative can have a critical impact on the nucleic acid quality. During this time, the tissue is starved of oxygen and significant protein and RNA degradation can occur within 10 minutes (Srinivasan et al. 2002). The time from procurement to fixation is referred to as warm ischemia time and should be minimized. Keeping samples cold (on ice) can slow autolysis, but immediate fixation ensures DNA and RNA content and quality.

If a sample is immersion fixed, the size of the sample is critical. Formalin penetrates tissue at a rate of 1 mm/h, however, penetration rates decrease with depth and a 5 mm piece of tissue requires 8 h for adequate fixation (von Ahlfen et al. 2007). If a sample is too large, the center will undergo significant autolysis prior to penetration with fixative. In general, to properly immersion fix a tissue sample, the sample should be 0.5–1 cm thick, in a 1:10 tissue volume to formalin volume ratio for 12–36 h (von Ahlfen et al. 2007) although a minimum time of 5 h is recommended for needle biopsy samples (Hewitt et al. 2008). Overfixation of tissue can lead to irreversible nucleic acid-protein crosslinks that preclude successful analysis while underfixed tissues may autolyse or undergo nucleic acid degradation.

Fresh frozen samples are an alternative to immersion fixation. Frozen tissues, either snap frozen in liquid nitrogen or embedded in Optimal Cutting Temperature (OCT) blocks, are used for gene and protein expression analysis in prospective stud-

ies. OCT is a water soluble embedding media comprised of glycols and resins that protects and supports the tissue during the freezing and cryosectioning process. Fresh frozen specimens are typically cut and fixed with an aldehyde fixative. The samples may be archived (at <−70 °C) until sections are needed. They may require little to no antigen retrieval depending on if the tissue is fixed. Frozen tissues are also typically more fragile than FFPE samples, and great care must be taken during sample handling. The freezing process is thought to preserve nucleic acids better than paraffin embedded samples (Wilcox 1993), presumably due to the speed of the freezing process relative to the penetration of fixative during immersion fixation for paraffin embedding. When compared to snap frozen tissues, OCT has been shown to negatively impact the quality of DNA and RNA in the tissues (Turbett and Sellner 1997)

The most common fixative used in toxicologic pathology is neutral buffered formalin (e.g. 10% NBF). Formaldehyde was discovered in 1859 (Howat and Wilson 2014) and its use in pathology is widespread. Pathologists are comfortable with the tissue morphology of formalin fixed specimens and it is unlikely to be replaced. Tissue archives, which serve as the source for retrospective analysis, are comprised of FFPE blocks.

Formalin is an aldehyde that reacts with amino groups resulting in the formation of methylene bridges, and is referred to as a cross linking fixative. These bridges can occur between proteins, between DNA and proteins, or between complementary strands of DNA. The resultant DNA trapping contributes to the decreased yield of DNA or RNA from the fixed tissue. While considered partially reversible, the amount of irreversible binding increases with time, resulting in decreased DNA or RNA yield from the tissue. DNA that has been cross linked to proteins or other DNA strands is susceptible to mechanical stress and DNA strand breaks. DNA strand breaks occur when the formic acid reacts with the purine bases, leaving them susceptible to cleavage and single strand breaks (Dietrich et al. 2013). Paraformaldehyde, another cross linking fixative, is used at the time of necropsy to perfuse tissues and can be used as a fixative for frozen sections, although it provides for much less tissue penetration compared to formalin.

Ethanol (and other alcohols) are coagulative fixatives. These fixatives denature the proteins and do not preserve the morphologic features of tissues adequately for histologic examination and are not commonly used for routine pathology studies. However, they collapse DNA and result in better yield for nucleic acid analysis. Acetone is commonly used as fixative for frozen sections. There are many other fixatives available that are used in pathology for special procedures or preserving specific tissues. As these are not commonly encountered in routine pathology studies or archives, they are not covered in this chapter.

12.2.2 Processing and Storage

The process of embedding tissue in paraffin begins with dehydration in a series of ascending alcohol concentrations. The tissues are then saturated with paraffin for 2–4 h at 65 °C. When performing ISH, paraffin embedding is one step that may contribute to

the loss of signal in FFPE tissues (Wilcox 1993). This may be due to the presence of nucleases or other contamination within paraffin wax, type of paraffin (natural vs. synthetic), or the temperature at which the samples were processed. Synthetic paraffin has a lower melting temperature and improved amount of nucleic acids recovered when compared to the higher melting temperature natural paraffin (Hewitt et al. 2008).

Once processed, FFPE blocks can be placed in storage indefinitely, however, degradation of nucleic acids increases over time. In general, to increase the likelihood of success in analyzing older samples, the smaller the target sequence should be (Greer et al. 1994). In addition to the time in storage, storage temperature can cause significant differences in RNA yield. The higher the storage temperature, the greater the degree of degradation observed. When performing a retrospective study in which the sample preparation is either unknown or may not be optimal, amplifying smaller PCR products (100–300 bp) result in greater success than larger fragments (Groelz et al. 2013; von Ahlfen et al. 2007; Scicchitano et al. 2006).

12.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) has been used for diagnostic purposes since the mid-1980s. In depth discussion of principles and methodology is beyond the scope of this chapter and can be found in a number of review articles (Garibyan and Avashia 2013; Bej et al. 1991).

The basic premise of PCR is that in the presence of DNA polymerase, free nucleotides, and primers, a DNA template can be amplified exponentially. The DNA amplified depends upon the primer specificity and location. Primers are short chemically derived sequences of DNA that are complementary to the region of interest. The template can be DNA or RNA, although RNA will require the additional step of generating complimentary (cDNA) using reverse transcriptase followed by PCR (RT-PCR). The amplification repeats in a cyclical fashion until the DNA product (amplicon) becomes detectable.

Quantitative PCR (qPCR) is also referred to as Real Time PCR (Heid et al. 1996). qPCR provides a method for extrapolating the amount of template DNA in a given sample. Originally used primarily for DNA quantitation (viral load) (VanGuilder et al. 2008), it is now widely used to study gene expression and validate expression of biomarkers (Winer et al. 1999). With qPCR, the product is measured in "real time" using a fluorescent probe. Probes can be nonspecific dyes that bind to double stranded DNA or sequence specific. qPCR has multiple advantages over basic PCR including the ability to quantify the template DNA and elimination of post PCR manipulation (gel electrophoresis) (Table 12.1).

In drug discovery and development, PCR can be used to detect loss or presence of gene expression in a given tissue, presence of mutated genes (Cerutti et al. 1994), identification of targets of possible therapies, characterization of biomarkers to better define prognosis, and for the identification of etiologic agents (i.e. viruses). Examples of PCR in oncology are abundant (Crocker 2002). RT-PCR, which relies on the

Table 12.1 PCR methods and definitions

Abbreviation	Definition	Template	Common uses	Output measured
PCR	Standard polymerase chain reaction	DNA	Cloning and genotyping	At the end of the PCR cycles, presence of product of interest is measured using intercalated DNA dyes, gel electrophoresis (semi-quantitative)
RT-PCR	Reverse transcriptase polymerase chain reaction	Single stranded RNA is reverse transcribed to single stranded DNA (cDNA) prior to the PCR	Gene expression mapping and cloning	An additional step and additional variable, to standard PCR. Measured as above. (Semi-quantitative)
qPCR or qRT-PCR	Quantitative or quantitative real time polymerase chain reaction	Single stranded DNA (or RNA if a reverse transcription step is included)	Gene expression analysis, companion diagnostics	The quantity of end product is measured in "Real time" rather than waiting until the end of the cycles. Measured against standards, the quantity of the template can be calculated. (quantitative)

reverse transcription of an RNA template to generate cDNA prior to PCR, was used to detect the presence of the fusion gene AML1/ETO in Acute Myeloid Leukemia (Kwong et al. 1995). Once the gene was identified, its presence or absence became a marker of response to treatment. Its quantification using qPCR was used to detect the presence of minimal residual disease (Marcucci et al. 1998). PCR) is used to detect mutations in oncogenes or tumor suppressor genes (Loda 1994), and identify causative agents of tumors or opportunistic infections (e.g. presence of Epstein Barr virus or Human Papilloma Virus in tumor samples) (Kiyabu et al. 1989).

qPCR is considered the gold standard for gene expression analysis (D'haene et al. 2010). In drug discovery and development, this is expanded to provide an overview of the gene expression levels genes involved in drug metabolism (CYP enzymes and peroxisome proliferators for example) in response to test article administration (Gerhold et al. 2001). These data, in conjunction with the histologic examination, can elucidate mechanisms of toxicity and/or help prioritize compounds of interest.

12.4 In Situ Hybridization

In situ hybridization (ISH) is a method used to detect specific nucleic acid sequences from DNA or RNA in cells, preserved tissue sections, or whole tissue. The method was largely pioneered through the experiments of Joseph Gall and Mary Lou Pardue, who used radiolabelled DNA on a cytological preparation (Gall and Pardue 1969). ISH employs the use of DNA or RNA probes that are complementary to a target

DNA or RNA of interest. The target probes may be either isotopically labeled or labeled with a hapten or fluorescent molecule. The probes are then hybridized to the tissue/cell preparation of interest and visualized via autoradiography, biochemical methods, or fluorescence detection. ISH is a powerful technique to examine chromosomes, mutations in DNA or RNA, and spatial and/or temporal gene expression in a variety of different sample types including FFPE or fresh frozen samples from tissues or cells. ISH may be a secondary method used to demonstrate target RNA when IHC for protein does not work. ISH is also extensively used in clinical applications for diagnostics purposes such as the HER2 ISH assay to test for breast cancer (Slamon et al. 1987). More recent applications have utilized ISH to examine the pattern of small RNA molecules called micro-RNAs (miRNAs), which are non-coding RNAs that function in RNA silencing and regulation of gene expression. Some of the important concerns for designing ISH experiments for drug discovery and development are discussed below.

12.4.1 Probe Design and Label Selection

Multiple methods for conducting ISH exist including using complementary DNA (cDNA), RNA (cRNA), or synthetic oligonucleotides as probes. When choosing a probe, probe size, and labeling method should be considered as they are critical for probe penetration into the tissue and determining proper hybridization conditions

Traditional methods of labeling ISH probes rely on using radioactive labels (e.g. ^{3}H, ^{32}P, ^{33}P, or ^{35}S) for high sensitivity. Advances in technology and science have included fluorescent, biotinylated, enzymatic, or hapten conjugated probes. Another popular method employs branched DNA technology. Branched DNA probes rely on signal amplification that has become a powerful and sensitive method for ISH. The advantages and disadvantages of these labeling methods are discussed in more detail in the following sections.

12.4.1.1 Probe Generation—Traditional Approaches

Synthesis of cRNA probes (riboprobes) may be performed through standard molecular biology techniques such as in vitro transcription or nick translation (Kumar 2010). In vitro transcription is the most common platform which utilizes a cDNA sequence as a template. Different initiation sites at either end of the sequence allow for generation of the anti-sense strand or sense strand probe. The anti-sense strand probe will hybridize to the target (mRNAs are oriented in the sense orientation) while the sense probe may serve as a negative control as it should not bind to the sense strand (e.g. mRNA target). The template is then incubated in a reaction mixture containing labeled nucleotides. One of the nucleotides is labeled with a radioisotope such as ^{35}S or with a hapten such as biotin, digoxigenin, fluorescein, or bromodeoxyuridine (BrdU). ^{35}S-labeled probes are detected with x-ray film/autoradiography, while the use of haptens relies on histological or immunohistochemistry detection systems.

Nick translation is another method to generate labeled probes. In this approach, the DNA template of interest is treated with DNase to create single strand "nicks" across the phosphodiester backbone of each DNA strand. The reaction is then incubated with DNA Polymerase I, which has exonuclease and polymerase activity. The exonuclease activity of the enzyme removes the "nicked" DNA bases and its polymerase activity replaces them with any labeled nucleotides present in the reaction. Both isotopic and nonisotopic labels can be used with this approach and the researcher can vary the concentration of labelled nucleotide in order to alter the amount of label incorporated into the probe (Kumar 2010).

12.4.1.2 Radiolabeled Probes

Isotopically labeled probes have been traditionally used for ISH and are considered among the most sensitive methods of detection. Synthesis of a radiolabeled probe is commonly done using an in vitro transcribed RNA from a DNA template in the presence of a radioactively labeled nucleotide triphosphate. Once synthesized and used for hybridization experiments, the tissue is further processed and the signal detected by autoradiography. Several protocols for preparing radioactively labeled probes are described in more detail here (Wilcox 1993).

Many researchers believe that radiolabeled probes are the most sensitive detection reagents for ISH due to the ability to amplify signal by prolonged incubation times, but the technology for using nonisotopically labeled probes has progressed substantially and is widely viewed as equally sensitive (see the following sections for a more detailed description) (Jensen 2014). Radiolabeled probes also can be synthesized from nearly any DNA template via in vitro transcription, provided a suitable promoter region for RNA polymerase is available for the reaction.

Problems associated with using radiolabeled riboprobes include the instability of the radioactive label and the tendency of the RNA to degrade over time (Levsky and Singer 2003). Several in vitro transcription reactions might be required for one experiment which could decrease the reproducibility of the assay or lead to inconsistent results. The efficiency of incorporation of the radioactive label may also vary between reactions depending on the quality of the template/reagents or the age of the radiolabeled NTP. Use of radiolabeled probes may also lead to problems with poor resolution of images and/or long exposure times to film or autoradiography screens (Levsky and Singer 2003). High background associated with exposure of the labeled tissue may also complicate experiments with these probes.

12.4.1.3 Hapten-Conjugated Probes

Nonisotopically labeled probes may be conjugated with haptens such as digoxigenin, biotin, or fluorescein and in vitro transcription kits for labeling RNA probes with haptens are commercially available. Synthesis of these probes is similar to radiolabeled methods except for the presence of a hapten-labeled NTP in the in vitro transcription

reaction mixture. After hybridization with a hapten-conjugated probe, IHC for the hapten is typically done with a specific antibody directed against the hapten.

Hapten-conjugated probes offer several advantages to traditional isotopic probes. First, different hapten labels can potentially be used simultaneously for several different targets which allows for multiplexing in one tissue section. Second, use of enzymatic detection systems allows for chromogenic or fluorescence detection that may be detected in a matter of hours rather than the days that might be required for exposure of isotopically labeled probes. Chromogenic detection systems have also become quite advanced, and using standard IHC detection chemistries allows ISH results that provide superior images and tissue morphology as compared to radiolabeled methods. Multiplexing ISH targets with protein targets by IHC can also be routinely achieved and is quite useful when the researcher wants to examine RNA and protein distribution in the same cell(s) (Fig. 12.1). Finally, some companies synthesize custom DNA-based probes conjugated to a hapten of interest.

Loss of sensitivity compared to radiolabeled probes is one of the challenges of using hapten labeled probes, however, Tyramide Signal Amplification (TSA) systems are available to overcome this. TSA systems employ the use of a tyramide molecule that is labeled with a hapten or flouorescent dye (Kerstens et al. 1995). In the presence of HRP (e.g. HRP-conjugated antibody), the labeled tyramide is covalently linked to the target of interest in areas only where the HRP enzyme is bound. Once the tyramide molecules are bound, this allows subsequent detection by chromogenic or fluorescent methods with a stronger signal than without TSA. TSA is extremely sensitive as it allows for the addition of multiple tyramide molecules to be bound to the target of interest which results in a greater likelihood of detection.

12.4.1.4 Fluorescent Labeled Probes

Fluorescent in situ hybridization (FISH) is a powerful cytogenetic approach that has traditionally been used to assay for the presence/absence of specific DNA sequences in chromosomes. FISH Imaging has typically utilized fluorescence microscopy. Probes for FISH have been available since the 1970s and are extremely useful for multiplexing different ISH targets in the same tissue. Technological advancement of the technology has even led to reports of FISH technology for single RNA transcripts (Femino et al. 1998).

Directly labeled FISH probes are attractive in that they may be more cost-effective than some of the hapten conjugates or the branched DNA technology (discussed below). Directly labeled FISH probes may also promote less complex staining protocols as the researcher also would not require chromogenic or fluorescent detection systems. The ability to easily multiplex targets is arguably the greatest benefit of using directly labeled FISH probes.

Background fluorescence, either from nonspecific binding or tissue autofluorescence, is considered one of the biggest challenges associated with FISH. Autofluorescence is common, especially in FFPE tissue, which may preclude the use of fluorescent probes unless a signal amplification system is used.

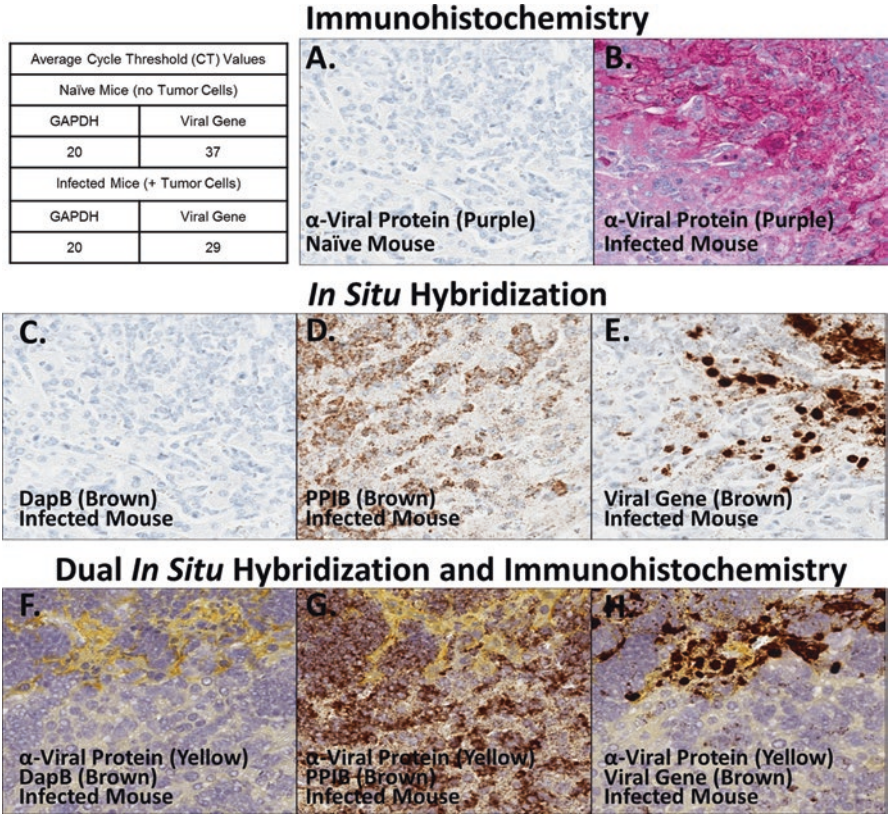

Fig. 12.1 Examples of Immunohistochemistry (IHC) and ISH. IHC and ISH were performed on FFPE mouse liver implanted with tumor cells infected with a murine virus (**a**–**h**). The table includes qPCR cycle threshold (CT) information for a housekeeping gene and the viral gene of interest for the tissues examined. IHC alone was performed to demonstrate the presence of viral proteins (**a**, **b**). Naïve animals not implanted with virus-infected tumor cells were negative for viral protein (**a**) while tumor-implanted animals were strongly positive at or near the site of tumor growth (*purple*; **b**). Next, ISH was performed on naïve animals (data not shown) or on animals implanted with infected tumor cells to interrogate the tissue for the presence of viral mRNA (*brown*; **c**–**e**). DapB (**c**; negative control), PPIB (**d**; positive control), and a viral probe (**e**) were used. Note the strong PPIB signal in tumor cells as compared to surrounding tissue. Also note the dissemination of virus from tumor cells to neighboring hepatocytes in (**e**). Finally, dual IHC and ISH were performed on naïve animals (data not shown) or on animals implanted with infected tumor cells to examine if viral mRNA (*brown*) and proteins (*yellow*) co-localize (**f**–**h**). Note that levels of viral proteins are readily visible in tissue probed with DapB (**f**) while PPIB expression appears mostly outside areas of active infection (**g**). Viral protein and mRNA appear to strongly co-localize (**h**)

12.4.1.5 Branched DNA Probe Technology

Branched DNA (bDNA) probes rely on signal amplification methods for visualization of the target nucleic acid. bDNA assays utilize target probes that are bound to pre-amplification sequences. Target probes are first bound to the target. There can be multiple target probes (~ up to 20) within a sequence of interest. Amplifiers are

then bound to the pre-amplification sequences that are bound to the target. Labeled probes are then bound to the amplifiers for subsequent detection. Typically the labeled probes are bound with enzymes (HRP or AP) for chromogenic detection, but may be bound to fluorophores for fluorescent detection. The bDNA method is extremely sensitive, and is hypothesized to allow up to 8000 labels for each nucleic acid molecule (Wang et al. 2012; Kwon 2013).

bDNA technology is extremely sensitive and specific, and some researchers have proposed that the method can theoretically detect single mRNA molecules (Wang et al. 2012). In practice, this may not be entirely accurate, and it is likely that at least 10–50 copies of a particular transcript/cell will be required for adequate detection.

12.4.2 Validation Strategies and Selection of Controls

Validation of any ISH method used with proper controls is critical. Along with examination of a target DNA or RNA, appropriate positive and negative controls that assess tissue quality and the presence of the target should be included. Assays to evaluate tissue quality are typically done with housekeeping genes (e.g. poly-T or beta actin) as positive controls. (Fig. 12.2) Housekeeping genes are usually expressed in most tissues of interest.

Negative control probes are equally important as the positive controls. Probe design will depend on the particular ISH platform being used, but traditional negative controls for oligonucleotide-based ISH assays include "scramble" or "sense" probes. Scramble probes contain nonsense sequences that have been validated not to bind target nucleic acids. Sense probes are designed to be the same sequence as the target, which will thus bind to the sense strand of the mRNA. As the true ISH probe binds in a reverse complementary fashion to a target nucleic acid, only the antisense probe

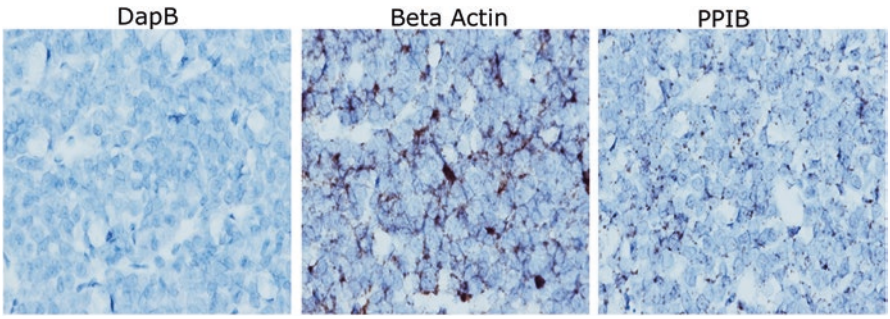

Fig. 12.2 In situ Hybridization (ISH) in Rat Pituitary. ISH was performed on a FFPE rat pituitary using branched DNA amplification (RNAscope®). ISH was performed according to manufacturer's recommendations using an automated staining platform. DapB (negative control probe), β-actin (moderate-strong positive control probe), and PPIB (low-moderate positive control probe) are shown in the panels above. Hematoxylin was used as a nuclear stain. Note that the size of the *punctate dots* is an indicator of gene expression level (e.g. compare strong expression of β-actin with the lower expressed PPIB transcripts)

should bind to the target. Use of the sense probe should result in no binding and is a common strategy used for a negative control. Finally, some assay platforms typically use a negative control that is an *E. Coli* target (DapB) which is not present in most samples. As the branched DNA method is a fairly sensitive technique, more moderately expressed genes (e.g. Peptidylprolyl Isomerase B; PPIB) are typically run as positive controls (Wang et al. 2012).

12.5 Next Generation Sequencing

Next-generation sequencing (NGS), also known as high throughput sequencing, is a general term used to describe a number of different sequencing technologies that have greatly reduced the cost and time for DNA or RNA sequencing (RNA-seq). In summary, sequencing is massively performed in parallel on overlapping fragments of nucleic acid. The raw fragment sequence data must be evaluated and processed for quality and computationally assembled into longer sequences up to and including entire genomes. This processed data now becomes the raw data for bioinformatics analysis that ideally yields insight into appropriately designed experimental questions. The role of the pathologist may be to contribute to experimental design, confirm biological phenotypes based on morphologic observations, contribute to sample stratification, and ultimately contribute to interpretation and application of the bioinformatics data to the original samples groups.

Transcriptome analysis may be addressed by expression microarrays or RNA-seq. Expression microarrays use thousands (or more) of predetermined probes that match complementary sequences within the sample thereby generating a profile of transcripts being expressed. It is limited by the probe library which targets known sequences and will miss rare or untargeted RNA species. Microarray expression is most useful when target transcripts are known. RNA-seq is more comprehensive. DNA can be sequenced followed by assessment of the presence and quantity of RNA transcripts within a sample at a given time point. This yields information on RNA processing, alternate splicing, rare species, and transcript modifications including mutations and single nucleotide polymorphisms (SNP). Transcript profiling can include total RNA, mRNA, tRNA, miRNA, and ribosomal profiling. As with all large complex data sets, computational challenges are present and nuance of final data interpretation requires team input, including pathologists.

12.6 Examples of Molecular Techniques Added Value in Drug Development

Immunomodulatory drugs are currently in development or marketed for disorders of the immune system like Rheumatoid Arthritis, Psoriasis, and Inflammatory Bowel Disease. During development, testing of these compounds in animals can

produce unexpected safety concerns as a result of their immunomodulatory characteristics. The pathologist and toxicologist must utilize their knowledge of pharmacology to determine if the effect is immunotoxicity or immunomodulation (Haley 2012). Subtle effects of subclinical infection are understood in small animals used in preclinical safety studies but also play a role in studies utilizing nonhuman primates (Lerche and Osborn 2003). In particular, nonhuman primates pose a particular challenge as they harbor a number of viruses, bacteria, and parasites that can alter immune function or can cause morbity and mortality under conditions of immunosuppression (Saravanan et al. 2015; Price 2010). PCR and antibody screening of the animals for pathogens prior to inclusion in studies is critical. PCR, IHC, and ISH are important tools in the diagnosis of opportunistic infections. For example, simian retroviruses can alter cytokine expression and immune cell function, cause organ weight changes, histologic changes, and increase susceptibility to infection and tumor development (Lerche 2010).

Inhibition of T cells signaling and proliferation by alefacept in nonhuman primates resulted in gammaherpesvirus B-lymphocyte proliferation (lymphoma). ISH was used to demonstrate lymphocryptovirus (LCV) in the affected tissues in the animals.

Polyomaviruses have potential implications in preclinical safety assessment. Simian Virus 40 shares 69% homology with the human JC virus, has similar biology and can cause similar disease. In the immunocompetent host, the virus remains latent. In the immunocompromised host, it replicates in oligodendrocytes and causes a progressive multifocal leukoencephalopathy (PML). Most commonly, this is reported in SIV infected macaques or HIV infected humans. PML in humans has also been associated with use of rituximab, natalizumab, and efalizumab. In situ hybridization can be used to confirm the presence of the polyomavirus within the lesions (Horvath et al. 1992). To our knowledge, there have not been published reports of simian PML associated with immunomodulatory therapy. While PCR, IHC, and ISH can detect the presence of pathogenic organisms, it is incumbent upon the pathologist to interpret molecular findings in conjunction with morphologic change to determine relevance to disease pathogenesis.

12.7 Utility of Molecular Techniques as Companion Diagnostics

With the push toward personalized health care and the aim of the right patient with the right drug at the right dose at the right time, molecular pathology is an integral part of the developing a sound biomarker strategy. The discovery of HER2 and its use as a biomarker is a prime example of how molecular techniques can help develop a biomarker that has a significant impact on human health. Human epidermal growth factor receptor-2 (HER2/neu, c-erbB2) was amplified in a human breast cancer cells and its overexpression was shown to have a significant impact on prognosis (Slamon et al. 1987). The direct link between HER2 overexpression and clinical outcome

made it a target for intervention. The humanized monoclonal antibody trastuzumab revolutionized care for HER2 positive breast cancer and was a breakthrough in personalized medicine. FISH is an FDA approved companion diagnostic for the stratification, prognosis, and treatment of HER-2/NEU in breast cancer. (FDA.gov)

Real time PCR is utilized as a companion diagnostic in that can identify genetic mutations that make a particular tumor sensitive to a therapy. Gefitinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that is particularly effective in non small cell lung carcinoma with deletions in exon 19 or 21 (Takano et al. 2008) FDA approved companion diagnostics utilize qPCR to detect mutations in the EGFR. PCR based companion diagnostics are also utilized to identify BRCA1 and 2 mutations, and KRAS mutations. (FDA.gov)

12.8 Summary

Molecular pathology is a rapidly progressing field. Pathologists are increasingly being asked to use these tools and participate in the interpretation of molecular data. Basic understanding of the techniques, their usefulness and their limitations is necessary to be a valued, contributing member of the multidisciplinary investigational team.

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Chapter 13
Current Issues in Developmental Immunotoxicity

Jamie C. DeWitt and Deborah E. Keil

Abstract Developmental immunotoxicity (DIT) occurs when early-life events reshape immune development to the point of altering immune system responses, often with negative consequences for an organism. Within the vein of toxicology, such events include exposure to exogenous risk factors, including chemical, biological, or physical agents that change the course of immune system development. Early-life exposure to such events may result in suppression, hyperactivation, and/or misregulation of a host of immune responses to result in inflammatory diseases, allergy and asthma, autoimmune diseases, and/or decreased resistance to infectious agents. This chapter will review current issues associated with DIT, including an overview of current guidelines for assessing DIT in experimental animal models, lifetime health risks thought to be associated with DIT, the need for DIT testing in safety assessments of chemical substances, and the impacts of "twenty-first century toxicology" on DIT approaches and methods.

Keywords DIT • Immunosuppression • Hyperactivation • Misregulation • Early-life exposures • Critical windows

13.1 What Is Developmental Immunotoxicity (DIT)?

13.1.1 DIT Defined

Developmental immunotoxicity (DIT) occurs when early-life events reshape immune development to the point of altering immune system responses, often with negative consequences for the lifetime of the affected organisms. It is generally

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accepted that the developing immune system is more sensitive to exogenous stressors, including xenobiotics, than is the fully developed immune system (Luebke et al. 2006) and numerous studies with experimental animal models indicate that compromised immunological development increases susceptibility to infection throughout life (Dietert and DeWitt 2010). Typically, common infectious diseases, such as otitis media, are hallmark indicators of DIT in human populations. However, Hessel et al. (2015) recently noted increases in the prevalence of juvenile onset allergic, inflammatory, and autoimmune diseases over the past decades, suggesting a role for DIT in human populations. Several reviews of DIT over the past few years have emphasized a role for DIT in a host of human disease patterns that emerge in early life. DeWitt et al. (2012a) summarized a series of review articles by Dietert and Zelikoff (2008, 2009, 2010) that identified four main patterns of pediatric immune-related diseases—allergic, autoimmune, inflammatory, and infection-related—that are associated with later-life immune-based diseases. In a follow-up review by Dietert et al. (2010) and in the review by DeWitt et al. (2012a), the authors suggested that a key to managing and reducing these later-life immune-based diseases was recognizing and treating the underlying early-life immune dysfunction. In addition, the authors emphasized that DIT testing should be a required component of safety testing for drugs and chemicals and that the endpoints evaluated should be *directly* relevant to immune-based diseases. Therefore, the purpose of this chapter is to present current issues in DIT to determine if and how testing has been improved to work to reduce immune-based diseases.

13.1.2 Current Methods of Evaluating DIT

As the developing immune system is considered significantly different from the adult immune system (Holsapple et al. 2004), evaluation of DIT must account for this difference to ensure that the most appropriate endpoints, for the age of the organism, are assessed. Recently, DeWitt et al. (2012b) outlined optimal developmental exposure scenarios and assays for detecting immunomodulation in experimental rodent models. Briefly, the authors suggested that a basic protocol for evaluating DIT include sufficient breadth and diversity to assess a range of immune functions to capture the entire risk for the immune system. Such a protocol, at minimum, should begin with an in utero exposure no later than gestational day 6 (GD6), which is sufficiently early in development to ensure that the test agent is present when hematopoiesis begins at GD7. DeWitt et al. (2012b) then indicated that exposure could: (1) occur for the duration of gestation only, ending end at birth; (2) occur through weaning of the offspring for both gestational and lactational exposure; or (3) include gestation, lactation, and the entire juvenile period (Fig. 13.1). Regardless of the exposure scenario, DeWitt et al. (2012b) recommend assessing immune parameters at 6 weeks of age, which is generally accepted as the age of immune competence for experimental rodent models. At this age, a full range of immunological assays can be performed and should, at minimum, include a combination of

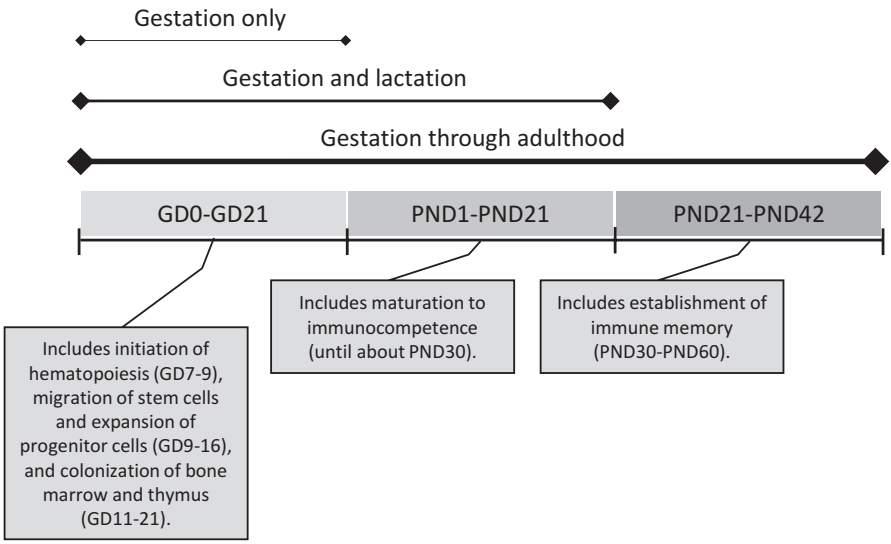

Fig. 13.1 Basic protocols for assessment of developmental immunotoxicity (DIT) in a rodent model as outlined by Dietert and Holsapple (2007) and DeWitt et al. (2012b). Note that exact timing of events may fluctuate somewhat from protocol. Gestational exposure may begin with pairing of male and female breeders as much as 7 days prior to the onset of gestation and end at GD20 or GD22 based on slight variability in gestation. Similarly, weaning may occur after PND21, depending on the ability of offspring to survive without maternal care. Offspring generally are considered immunocompetent as early as PND42, but are generally not considered "adults" until about 8 weeks of age. GD: gestational day. PND: postnatal day

observational and functional assays. DeWitt et al. (2012b) recommend that observational assessment include immunophenotype, histopathology, and/or lymphoid organ weights and that functional assessments include measures of adaptive immunity (i.e., T cell-dependent antibody responses or delayed-type hypersensitivity) and innate immunity (i.e., natural killer cell or cytotoxic T cell assays). These particular assays for assessment of immunocompetence have been reliably and repeatedly used in experimental rodent models and also demonstrate relatively robust concordance with similar assays applied to humans (Selgrade 2007).

Collinge et al. (2012) considered DIT testing in the context of pharmaceutical product development, rather than in the context of industrial or environmental chemicals. In this review, the authors emphasized that while DIT considerations have been part of pharmaceutical product applications since the early 2000s—when immunotoxicity occurs in adult animals for agents that are to be given to pregnant women—the methods by which DIT is evaluated have not yet been harmonized. Additionally, Collinge et al. (2012) pointed out that for pharmaceutical agents, the exposure scenario to the offspring is likely to be well defined as gestational and/or lactational or direct, and therefore assessments could be limited to these scenarios. Other than considerations regarding the exposure scenario, Collinge et al. (2012) and DeWitt et al. (2012b) were in agreement with regard to the suite of assays that should be included in an assessment of DIT. Importantly, neither of the authors

indicated that alternatives to in vivo testing were sufficient to capture impacts on immunocompetence; in vitro methods were not even mentioned in DeWitt et al. (2012a, b). Collinge et al. (2012) suggested that a battery of in vitro tests would be necessary and that alone, they would never be sufficient for evaluation of DIT.

Hessel et al. (2015) recently published a very interesting review of nine different industrial/environmental compounds and asked "are standard tests of developmental toxicity sufficiently sensitive to protect the developing immune system?" They asked this question as while DIT is still not a routine endpoint required for safety testing of non-pharmaceutical agents, assessments of developmental toxicity are required. Using compounds for which extensive DIT testing had been performed, that included exposure during the early postnatal developmental period, and included sufficient data for derivation of a benchmark dose (BMD), Hessel et al. (2015) concluded that DIT parameters were among *the most sensitive* parameters for each of the nine compounds. Further, Hessel et al. (2015) found that for three of the compounds, current intake levels for safety might need to be reconsidered as assessment of DIT indicated that these intake levels are likely insufficiently protective.

In summary, there is agreement among researchers that current methods for evaluating DIT need to include an exposure scenario appropriate for the test agent, whether it is a pharmacological agent or an agent of industrial use or environmental concern. There also is agreement that such methods need to include both appropriate observational and functional assessments of immunocompetence, as described in DeWitt et al. (2012b). Finally, it is clear that to sufficiently protect the public from potential health risks of exogenous agents, especially those with industrial and/or environmental concerns, DIT testing needs to be a routine part of all chemical safety assessments.

13.2 Critical Windows of Immune System Development

13.2.1 Overview of the Developing Immune System

13.2.1.1 Early-Life Risk

For 2000, it was reported that cause of death is lacking for 98% of the world's 4 million neonatal deaths. In a study by Lawn et al. (2006), these authors highlighted the lack of reliable cause-of-death data. However, based on best estimates for 193 countries, of the reported causes of neonatal death, the major known causes were estimated to be 38% infections (sepsis/pneumonia, tetanus, and diarrhea), 28% preterm birth, and 23% asphyxia (Lawn et al. 2006).

Approximately 10 years later, infection remains a leading cause of death in children. Recent reports specified that of the 6.3 million children who died before 5 years of age in 2013, 51.8% (>3 million) died of broadly-defined infections with the majority not living past the neonatal stage (Liu et al. 2015). In addition to infection, preterm birth complications, pneumonia, and intrapartum-related complications contributed to death.

Based on current trends, these authors estimated that 4.4 million children younger than 5 years of age would still die in 2030. With these statistics and projections, it is critical to address underlying causes of childhood morbidity and mortality.

Not represented in these statistics are the underlying causes of neonatal and childhood morbidity and mortality. In the past 70 years, the use of industrial chemicals has increased more than 15-fold (Federal Reserve Board 2015). People are now exposed to multiple environmental chemicals in the air, food, water, and in a variety of consumer products. Consequently, there are several examples of early-life exposure to environmental agents that cause immune suppression, hyperactivation, or other immune dysregulation, ultimately leading to inflammatory disease, allergy, asthma, autoimmune disease, or decreased resistance to pathogenic organisms. Therefore, prioritizing developmental immunotoxicology assessment strategies and regulatory policies is vital to informing prevention and reduction of early-life risk to infants and children.

13.2.1.2 Vulnerability of the Developing Immune System

This literature is backed by knowledge that the fetal time period is a critical window of immune system development (Luebke et al. 2006; Selgrade 2007; Dietert 2008; Dietert 2009; DeWitt et al. 2012a). There are documented increases in childhood immune-based diseases following environmental xenobiotic exposures during fetal and post-natal development (reviewed by Dietert 2014). Furthermore, a growing body of evidence supports the hypothesis that chronic diseases, including asthma, have fetal origins (Barker 1997; Barker 2001; Bateson et al. 2004; Ben-Shlomo 2007; Turner 2012).

13.2.1.3 Critical Stages of Immunological Maturation

Paramount to elucidating the effects of xenobiotics on the developing fetus and child, an understanding of the impact of exposure during critical windows of development is necessary. Exposure during different periods of fetal and neonatal development can result in striking and varied consequences on the immune system. Immunological maturation is organized by five major events: (1) initiation of hematopoiesis; (2) stem cell migration and cell expansion; (3) colonization of bone marrow and thymus; (4) maturation to immunocompetence; and (5) establishment of immune memory (Dietert et al. 2000; reviewed by DeWitt et al. 2012a). The first three phases, hematopoiesis, stem cell migration and cell expansion and colonization of bone marrow and thymus occur in the human fetus during gestational weeks 8–10, 10–16, and 16-birth, respectively (DeWitt et al. 2012a). Advances in immune competence occur during the lactational stage from birth to year one, while establishment of immunological memory continues from the first year to post-puberty age. When using rodent models for DIT assessments, gestation days (GD) 7–9 mark the initiation of hematopoiesis, followed by migration of stem cells and

expansion of progenitor cells during GD 9–16 (Dietert et al. 2000). GD 13 until birth is the period of colonization and establishment of the bone marrow and thymus (reviewed by Landreth 2002). A substantial portion of immune system development in rodents occurs postnatally (*see* Chap. 4). The DIT working group (Luster et al. 2003) emphasized that independent of the treatment route, exposure through PND 42 is required to accommodate the entire period of immunologic ontogenesis in rodent models.

13.2.1.4 Epigenetics and Its Role in Immunological Development

Throughout our life span, humans are exposed to a variety of chemicals that can cause deleterious effects to DNA such as mutagenesis or physical damage. However, recent studies are characterizing more subtle changes at the level of epigenetics. The term "epigenetics" encompasses stable yet reversible alterations in gene expression arising from variations in DNA methylation and histone modifications without altering the underlying DNA sequence. Also considered an epigenetic mechanism is gene silencing by small noncoding RNAs that include microRNAs (miRNA). Studies in vivo and in vitro have shown that xenobiotics are capable of changing the epigenetic pattern as well as miRNA expression in certain cell types.

Although there is much to be learned about the interconnection of epigenetics, xenobiotics and developmental effects, current and notable examples demonstrate its increasing implications. For instance, prenatal exposure to ethanol lead to the development of fetal alcohol syndrome that is characterized by mental retardation, behavioral problems, poor growth, craniofacial, cardiovascular, skeletal defects, and immune deficiency (Ornoy and Ergaz 2010; Tonk et al. 2013). The exact pathogenetic processes are unknown, but recent studies identified a mechanistic role for miRNAs induced at ethanol exposures corresponding to social drinking (Sathyan et al. 2007). Following low dose of bisphenol A (BPA) during gestation, brains from embryos (GD 18.5) exposed to BPA had lower gene transcript levels for several estrogen receptors, oxytocin, and vasopressin as compared with controls. A decrease in vasopressin miRNA persisted into the F_4 generation, leading to suppression of oxytocin in males (Wolstenholme et al. 2012). Another example includes toll-like receptor (TLR)-4-mediated stimulation of mouse macrophages by bacterial lipopolysaccharide (LPS) that triggered the differential epigenetic reprogramming of genes driving the inflammatory response (Arbibe and Sansonetti 2007).

Throughout in utero development, the placenta is a vital regulating barrier for the fetus, controlling growth and development through the transfer of nutrients and waste while offering some protection from xenobiotic insults (Robins et al. 2011). Of particular interest are placental genetic and epigenetic profiles that are linked to fetal development (Filiberto et al. 2011). For instance, repetitive genomic sequences such as LINE-1 and AluYb8 in the placenta exhibited increased methylation and were significantly and positively associated with birth weight among infants exposed to tobacco smoke and alcohol (MacGillivray and Kollman 2014). Heavy metals may also cross the placenta, compromising fetal development (Gundacker

and Hengstschläger 2012; Vahter 2009). However, there is little known about placental metal toxicokinetics. Metallothionein is likely responsible for placental storage of metals, especially to include cadmium, yet the route or specific transporters facilitating the influx of metals into the fetus are unknown. Therefore, one might expect the discovery of epigenetic modifications to the developing fetus in future studies as epigenetic modifications are an emerging mechanism linked to metals exposure (reviewed by Arita and Costa 2009).

The epigenetic signature of immune cells may reflect the history of modifications due to xenobiotic exposure or other environmental factors. In depth reviews are available describing an overview of transcriptional and epigenetic control of T cells (Wilson et al. 2009; Kanno et al. 2012; Weng et al. 2012). Relevant to this discussion is that the balance between Th1 and Th2 immune responses evolves during sensitization to a chemical allergen and can be modified during early development. During gestation and the neonatal period the immune system exhibits a preferential Th2 bias (Wilson and Kollmann 2008). Th2-biased neonatal immune system has concurrently low Th1 responsiveness to developing self or maternal protein antigens (Prescott and Saffery 2011). Importantly, the Th1/Th2 imbalance correlates with near 100% methylation of the interferon gamma promoter in neonatal T-cells (Renz et al. 2011). Exposure to microbial proteins resets the immune system with demethylation of the interferon gamma promoter (Renz et al. 2011). Dietary factors and xenobiotic exposure may further modulate the establishment of the neonatal Th1/Th2 immune repertoire influencing immune and allergic responses throughout life.

Emerging evidence suggests lymphocyte dysregulation is linked to epigenetic mechanisms targeting RUNX3 gene expression (Prescott and Saffery 2011). Hypermethylation of RUNX3, a gene involved in lymphocyte regulation, is linked to increased allergic airway disease development and severity (Schaible et al. 2011). Methylation patterns affecting RUNX3 gene were significantly associated following exposure to maternal cigarette smoking during pregnancy, yet another example underscoring the necessity to define epigenetic mechanisms in DIT (Maccani et al. 2013).

13.2.1.5 One Xenobiotic, Multiple Mechanisms of Action

DIT produces innumerable effects. Exclusive to DIT health effects is the timing of exposure that will dictate the immunotoxicological outcome. Even though a mechanism may be previously characterized for a xenobiotic in an adult model, this will not necessarily translate to DIT outcomes. Lead (Pb) is an example of an immunotoxicant that provokes different immunotoxicological effects depending on temporal exposure. During the perinatal period, Pb interferes with the seeding of the thymus and thymocyte maturation (Dietert et al. 2004). Rats given Pb late in gestation experienced the Th1/Th2 shift, whereas those given Pb early in gestation experienced a different DIT profile that precluded the Th1/Th2 shift (Bunn et al. 2001). Furthermore, if Pb exposure occurs throughout gestation, the juvenile and adult

delayed-type hypersensitivity (DTH) response is decreased. Pb exposure restricted of the first half of gestation leads to impaired macrophage function but no alteration in DTH (Dietert et al. 2004). Evidently, temporal exposure modifies the mechanism of action. Defining critical windows is required to fully evaluate DIT thereby escalating the complexity for regulatory testing framework that considers toxicant exposure effects across life stages (Daston et al. 2004).

Moreover, Dietert (2009) distinguishes between causes of immune dysfunction and triggers of diseases in DIT assessments. He states that a cause of immune dysfunction is one that strongly alters the status of the immune system following xenobiotic exposure during key periods of immune maturation (Dietert 2009). Immune dysfunction is most likely expected during gestational development. Conversely, infectious agents are an example of a trigger for immune dysfunction trigger (Dietert 2009) and differentiated in the fact that when exposure occurs, they most likely uncover or facilitate the existence of an underlying immune dysfunction (Dietert 2009).

13.2.2 Examples from Established Developmental Immunotoxicants

Among persistent organic pollutants (POPs) of concern among young children are polychlorinated biphenyls (PCBs). PCBs exposure decreased antibody responses to childhood vaccinations manifesting in increased incidence of infections (Stølevik et al. 2013). An additional study corroborated this observation by identifying significant correlations between doubling of serum PCB concentrations with a 20% decrease in antibody levels (Heilmann et al. 2006, 2010). Conversely, prenatal and lactational exposure to PCBs in children has also been linked to an allergic sensitization or overactive immunological response (Grandjean et al. 2010).

Developmental exposure by age 5 to perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), two compounds in a class of substances known as per- and polyfluroalkyl substances (PFASs), resulted in a several-fold increase in illness by age 7 (Grandjean et al. 2012). Further investigation identified that serum antibody levels for diphtheria and tetanus were below protective levels in these children. Recently, a toxicogenomics connection between prenatal exposure to PFAS and impaired immune function in early childhood suggested a PPAR- and NF-κB-mediated mode of action (Pennings et al. 2015). These findings add to the available evidence that exposure to PFASs is immunotoxic in children and support regulatory policies to reduce exposures to these substances.

Other xenobiotics also show an association between early-life exposure and immune-related diseases. Early-life exposure to tobacco smoke increases the risk of childhood asthma, but also subsequently increases the risk of additional diseases in adulthood, including obesity, behavioral disorders, lung cancer, and allergies (Dietert et al. 2010). Arsenic-induced immune dysfunction causes long-lasting health effects in vulnerable infants and children. This has been demonstrated in

drinking water studies in highly exposed populations in both Mexico and Bangladesh. Elevated urinary arsenic levels correlated with impaired peripheral blood mononuclear cells (Soto-Peña et al. 2006), while Rocha-Amador et al. (2011) reported that elevated arsenic exposure in water is linked to increased apoptosis in peripheral blood monocytes. Furthermore, prenatal exposure to arsenic interfered with thymic T cell development (Ahmed et al. 2012).

Several studies demonstrate that disruption in effective oxygen species regulation is a likely route to the elevated risk in DIT. Postnatal pediatric use of paracetamol was linked to asthma particularly in children with genetic alleles associated that control oxidative inflammation (NAT2, Nrf2, and GSTP1; Kang et al. 2013). Therefore, characterizing immunotoxicological effects due to exposure of reactive oxygen species during key developmental stages in the ontogeny of the immune system would be key to determining safety exposure thresholds for xenobiotics that share mechanisms altering reactive oxygen homeostasis.

13.3 Lifetime Risks After DIT

13.3.1 Diseases with Known DIT Risk Factors

Non-communicable adult diseases with evidence of DIT and immune dysfunction include certain types of leukemias, allergies, asthma, various autoimmune diseases, dermatitis, psoriasis, rheumatoid arthritis, ulcerative colitis, and myriad diseases with an inflammatory component (Dietert 2014). Exogenous agents known to be risk factors for these diseases include industrial compounds that also exist as environmental contaminants, such as polycyclic aromatic hydrocarbons and PCBs. These later-life immune-based diseases are not always unforeseen, as dysfunctions in a developing immune system could be expected to persist through adulthood. In 2012, Dietert and Luebke edited a book entitled *Immunotoxicity, Immune Dysfunction, and Chronic Disease*. While the book focuses on chronic diseases and their immunological origins, an underlying theme is early-life immune dysfunction and its contribution to chronic diseases. For a more in-depth analysis of diseases with DIT risk factors, readers are encouraged to seek out the text by Dietert and Luebke (2012).

13.3.2 Disease Patterns Thought to be Associated with DIT

As mentioned in Sect. 13.1.1, four main patterns of pediatric immune-related diseases exist—related to allergic, autoimmune, inflammatory, and infection-related diseases, and are associated with later-life immune-based diseases. Over the past 10 years, Dietert and colleagues have published numerous articles exploring the linkages between DIT and these four main disease patterns. Of particular relevance to

this section is an article on fractal immunology and immune patterning (Dietert 2011a) and an article on DIT in chronic disease and cancer (Dietert 2011b). In the first article, Dietert (2011a) asserts that immune health starts in the womb and is interfaced across the aging process. Although immune dysfunction that occurs at any point along the continuum can increase the risk of certain diseases, immune dysfunction that occurs early in life has the potential to increase the risk of disease patterns, resulting in multiple comorbidities in a single individual. The application of fractals, or a mathematical organization and reduction of the immune system to parts related to the whole, can help to identify patterns within the immune system and their relationships to the environment and diseases. Dietert (2011a) suggests that this approach is necessary to reduce the immune health gap that currently exists between safety testing for drugs and other chemicals and risk of disease. The fractal approach to immunomodulation as suggested by Dietert is complemented by compartment-based enhanced immunohistopathology evaluation (*see* Chap. 10). In the second article, Dietert (2011b) maintains that because many immune dysfunction-based diseases arise in non-immune tissues and are treated and managed based on the presenting systems, the involvement of underlying immune dysfunction is chronic disease is severely underestimated. Dietert (2011b) strongly argues that safety testing of drugs and other chemicals must include DIT and that testing must be capable of detecting diseases that fall within one of the four main patterns of immune-related diseases (i.e., allergic, autoimmune, inflammatory, and infection-related). These are strong arguments that such testing should be part of nonclinical safety evaluations.

13.3.3 Monitoring the Impact of Early-Life Environmental Factors on the Immune System for Prevention of Disease Strategies in Old Age

For a specific example, low dose, early life exposure to the insecticide permethrin leads to alterations in Nurr1, NF-κB-p65, Nrf2, lipid peroxidation and GSH levels (Fedeli et al. 2012). Developmental exposure to permethrin induced a significant increase in Nurr1 and lipid peroxidation that is evident only later in aging rats. TNF-α and Rantes increased, while IL-1β, IL-2, IL-13 decreased in the oldest treated rats. Therefore, it is conceivable that inflammatory parameters are potential biomarkers for monitoring the impact of early-life environmental exposure that lead to health impairment later in life.

In a broader view, Halfon and Hochstein (2002) presented a new model termed lifecourse health development (LCHD) that defines health as a dynamic process beginning at conception and continuing throughout the entire lifespan. By providing a better understanding of vulnerable windows in overall health development, the LCHD model focuses on identifying protective factors early in the lifespan to shift

the emphasis from treatment in the later stages of disease to prevention in the earlier stages of life (Halfon and Hochstein 2002). As we gain more insight of the interconnection of epigenetics, xenobiotics, and developmental effects, these advancements will underscore the importance of considering gene-environment interactions in the etiology of complex disease from conception to old age.

13.4 Why Is There a Need for DIT Testing of Chemical Substances?

The concept of effects related to critical developmental stages in development is integral to the US Environmental Protection Agency's (US EPA) *Framework for Assessing Health Risks of Environmental Exposures to Children* (overviewed in Brown et al. 2008). Although not a regulatory requirement at this time, research focusing on developmental immunotoxicity (DIT) and its role in childhood disease is an escalating priority as evidenced by increased government- and industry-funded DIT research including the National Toxicology Program (NTP). DIT was also considered in regulatory developmental and reproductive toxicology (DART) protocols (Burns-Naas et al. 2008).

Immunotoxicity testing is only required in adult-aged animal species under the Federal Insecticide, Fungicide, and Rodenticide Act but not under the Toxic Substances Control Act (TSCA). With regard to TSCA, immunotoxicity testing is only required in circumstances when a xenobiotic alters immune organ mass or cellularity. Regrettably, this policy is shortsighted as there are several examples of immunotoxicants that do not alter immune organ mass or cellularity in adult animal models, yet cause immune impairment in adult animals. Furthermore, xenobiotics that do not cause immunotoxic alterations in adult models are not precluded from causing long-lasting, immune impairment during fetal, neonatal, or juvenile stages (Keil et al. 2004, 2009; Luebke et al. 2006; Peden-Adams et al. 2007, 2008). As current regulatory policy stands now, adult-defined "non-immunotoxicants" will never be tested for early-life health risk (Dietert and DeWitt 2010).

13.5 Toxicology in the Twenty-First Century and DIT

13.5.1 Tox21 Defined

In 2007, the Committee on Toxicity Testing and Assessment of Environmental Agents of the National Research Council published *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy* (NRC 2007). This report emerged over several years, from the input of representatives of many agencies and organizations, and from an apparent need to incorporate new technologies and broadly address chemicals, mixtures, outcomes, and life stages, reduce time and cost of testing, use

fewer animals, and improve the scientific robustness of health effects assessment. In a summary of the report, Krewski et al. (2009) emphasized that the report heralded a paradigm shift in toxicity testing from expensive and lengthy in vivo testing models to identifying and using in vitro toxicity pathways in human cells or lines. These new approaches would focus heavily on computational systems biology models to determine dose-response perturbations of toxicity pathways and pharmacokinetic models to extrapolate from in vitro findings to in vivo human blood and tissue concentrations. This vision and strategy would thus move toxicity testing away from a reliance on whole organism toxicity to pathway assays in computational systems biology models (Krewski et al. 2009). In 2008, the U.S. EPA, the U.S. National Toxicology Program (NTP), and the National Institutes of Health Chemical Genomics Center (NCGC) established a collaborative research program to determine whether high-throughput and computational toxicological approaches could actually produce data predictive of results from animal toxicity studies. It is hopeful that this approach could be used for chemical prioritization for expanded testing thereby improving and streamlining prediction of human health risks (Collins et al. 2008).

13.5.2 Tox21 and Developmental Endpoints

As could be anticipated, an explosion of review and opinion articles occurred after the publication of these reports (termed "Tox21"). However, to our knowledge, only a handful address testing strategies for developmental toxicity, only one specifically addresses immunotoxicity, and none address DIT. Seiler et al. (2011) addressed issues associated with developmental toxicity and suggested that embryonic stem cell (ESC) differentiation assays were a promising approach for reducing and potentially replacing whole animal models. Accumulating data indicate that ESC assays for developmental cardiotoxicity have been especially well developed, assays for assessing developmental neurotoxicity are growing, and other systems and processes (i.e., metabolism) are showing promise (Seiler et al. 2011). Similarly, Smirnova et al. (2014) summarized some of the approaches being considered to meet the Tox21 vision for assessment of developmental neurotoxicity, including adverse outcome pathways (AOPs) and Integrated Testing Strategies (ITS) that identify toxicity pathways from exposure to disease or adverse health effect. Smirnova et al. (2014) noted that while a 2013 European Food Safety Authority panel on developmental neurotoxicity (DNT) concluded that no in vitro tests currently exist that can appropriately substitute for in vivo DNT assays, technological advances in organotypic cell cultures, stem cell assays, and biological phenotyping are helping to prioritize chemicals for more in depth DNT testing. Some of the problems with current DNT approaches is that they are exclusively based on expensive and time consuming in vivo models that produce data that often are difficult to interpret, tests are only performed when outcomes of other tests trigger them, and results are rarely used for regulatory standards or

risk assessment (Smirnova et al. 2014). This is an important consideration to Tox21—many of the novel in vitro and computational approaches being developed are meant to serve as alerts for particular endpoints and prioritize compounds for additional testing.

The challenges of evaluating DIT under a Tox21 vision, like DNT, are related to chasing a sensitive moving target. As Smirnova et al. (2014) pointed out for DNT, the difference between neurotoxicity and DNT is the developmental component, thus is not a question of "is it toxic or not," but rather a question of dose and timing. Developing systems tend to be sensitive to lower doses than adult systems, and the types of toxicities that arise are highly dependent on the developmental stage at which exposure occurs. This issue is further supported by Hartung and Corsini (2013), who indicated that no in vitro test models exist for DIT, even for evaluation of hypersensitivity and immunosuppression, the primary focus of in vitro methods for evaluating immunotoxicity. Hartung and Corsini (2013) also outline some of the major limitations of in vitro tests for immunotoxicity evaluation in adult organisms. Many of the limitations are associated with the nature of the immune system itself and how it works as an integrated network of different cells and signals that exists in numerous organs. No single in vitro system is able to recapitulate the entire immune system, so any in vitro approaches would be limited to minimal interactions. At the developmental level, these challenges are multiplied as even single cell types can vary drastically based on the tissue in which they mature.

13.5.3 Tox21 and TSCA in the US

In June of 2015, the U.S. House of Represenatives passed the TSCA Modernization Act of 2015 (H.R. 2576), which was designed to update the 1976 Toxic Substances Control Act (TSCA). The U.S. Senate version, known as the Frank R. Lautenberg Chemical Safety for the Twenty-first Century Act (S.697), is set for a vote sometime in the fall of 2015. Briefly, TSCA gave the U.S. EPA regulatory oversight of the manufacture and sale of chemicals in order to protect the public from unreasonable risk of injury to health or the environment. Since its passage in 1976, TSCA has not been substantially updated and it has been largely criticized for being ineffective. One of the major limitations to effective regulation of chemicals under TSCA is lack of sufficient toxicological data to make appropriate decisions. The modernized U.S. House version received widespread bipartisan support and in terms of Tox21, does not appear to have a substantial impact on testing requirements. The Senate version, however, has the potential to drastically alter testing requirements for chemical substances. One of the proposed sections includes language that directs the Administrator, prior to adopting a requirement for testing using vertebrate animals, to take into consideration (1) toxicity information, (2) computational toxicology and bioinformatics, (3) high-throughput screening methods and the prediction models of those methods, and (4) scientifically reliable and relevant alternatives to tests on animals that would provide equivalent information. The bill also contains Criteria for Adapting or Waiving Animal

Testing Requirements, which would allow manufacturers or processors to adapt or waive animal testing for a substance or mixture if, among others, testing for a specific endpoint is technically not practical to conduct. Additional language exists in the S.697 (as of August of 2015) to implement alternative testing methods that are not based on vertebrate animals and that can reduce, refine, or replace the use of vertebrate animals, including toxicity pathway-based risk assessment, in vitro studies, systems biology, computational toxicology, bioinformatics, and high-throughput screening. Should this version of TSCA reform pass the Senate and be adopted as law in the U.S., there may be a strong push to develop alternative strategies for detecting DIT. This may largely come out of industries associated with chemical manufacture and process in harmonization with the U.S. EPA.

13.6 Alternative Methods of Detecting DIT

Currently no methods alternative to those listed and referenced in this chapter exist for detecting DIT.

13.7 What the Future Holds

Current DIT testing strategies do not appear to be adequate for detecting immune-based diseases that present in systems other than the immune system. Current methods for detecting DIT also are heavily focused on hypersensitivity and immunosuppression, do not have maximal relevance for non-communicable and chronic human diseases with a basis in immune dysfunction, and poorly address differences in susceptibility based on critical windows of immune system development. In addition, the world of DIT research is woefully underprepared to tackle a paradigm shift called for by Tox21 and potentially, TSCA reform. This is not to say that researchers themselves are inadequately trained or motivated, but that technologies for turning the complexities of the immune system into something other than a whole animal model are practically nonexistent. Part of this limitation is that even now, new discoveries related to the immune system are being made—large scale discoveries and not just new cytokines that play a specific role in a specific tissue. In June of 2015, Louveau et al. (2015) published data that for the first time, demonstrated the existence of a central nervous system lymphatic system. Before these data were published, it was assumed that the central nervous system lacked lymphatic vasculature! Until the complexities of the immune system throughout life are better understood, alternatives to in vivo systems are unlikely to deliver data appropriate for the protection of human health and the environment.

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Chapter 14
The Murine Local Lymph Node Assay

Ton van Huygevoort

Abstract Allergies make up a major global health problem. After dermal contact with allergens, susceptible individuals are prone to allergic contact dermatitis which consists of two stages: skin exposure causing immunological priming and the acquisition of skin sensitization. Up until 2002, the most widely used animal models for the evaluation of the skin sensitizing potential of compounds were guinea pig models containing the induction phase as well as the challenge phase to elicit a sensitization response. In 1986, the murine local lymph node assay (LLNA) was described by Kimber et al. as a method for assessing the contact sensitization potential of compounds. Since that time, the LLNA has undergone extensive evaluation and validation and is the only validated in vivo method for detection of skin sensitization. Focusing on the induction phase only, the murine LLNA does not have a challenge phase. The contact allergenic potential of a compound is evaluated by the cellular proliferation of the draining lymph nodes of mice following topical treatment of the ear flap with test compound. This stimulation reflects the induction of an immune response. The LLNA utilizes three concentrations allowing for the assessment of a dose response relation. In order to distinguish between irritant and allergic responses in the LLNA, several modifications and alternative endpoints for the LLNA have been investigated. Compared to the guinea pig studies, the LLNA reduces the number of animals, provides objective and quantitative endpoints and is less prone to subjective interpretation.

Keywords Allergic contact dermatitis • Skin sensitization • Animal model • Hazard identification • Alternatives

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Abbreviations

(Q)SAR	(Quantitative) structure-activity relationship
3H	Tritiated
ACD	Allergic contact dermatitis
AOPs	Adverse outcome pathways
DAE433	*N,N*-dimethylacetamid/acetone/ethanol (4:3:3)
DC	Dendritic cell
DLNs	Draining lymph nodes
DPM	Disintegrations per minute
DPRA	Direct peptide reactivity assay
EC3 value	The estimated concentration needed to produce a stimulation index of 3
EPA	US Environmental Protection Agency
GD	Guidance document
GPMT	Guinea pig maximalization test
hCLAT	Human cell line activation test
ITS	Integrated testing strategies
LLNA	Local lymph node assay
MAFF	Japanese Ministry of Agriculture, Forestry and Fisheries
MEST	Mouse ear swelling test
NIH	National Institutes of Health
NK	Natural killer
OECD	Organisation for Economic Cooperation and Development
OPPTS	The Office of Prevention, Pesticides and Toxic Substances
PBS	Phosphate-buffered saline
REACH	Registration, Evaluation, Authorization and Restriction of Chemical Substances
SC	Stratum corneum
SI	Stimulation index
TG	Test guideline
TH1	T-Helper 1
TH2	T-Helper 2

14.1 Introduction: Allergic Contact Dermatitis

Allergies make up a major global health problem. After dermal contact with allergens, susceptible individuals are prone to allergic contact dermatitis (ACD) or skin sensitization. Up to fifteen percent of all reported occupational illnesses are cases of ACD. There are even cases, rare as they might be, in which hypersensitivity to certain compounds triggers immediate life-threatening reactions—known as anaphylactic shock. All sorts of components in numerous consumer products (like

cosmetics or clothes and toys), as well as dermal drugs, may cause ACD. Different elements in our environment play an important role as well. When that environment (for example, our office) makes us perspire, sweaty skin makes powders, for instance, stick to the skin easily and the intake of allergens increases. Or when the air in our house is very dry, the dry air can cause our skin to chap to the degree that the epidermal surface is disrupted. A chapped or abraded skin will be far less able to protect against allergens. Tattoos, for that matter, also severely compromise the skin's protective barrier function since the ink is brought into the skin behind the stratum corneum.

Usually, ACD occurs as a result of repeated or prolonged contact with a compound that triggers the immune system. Its development consists of two stages. Stage one—skin exposure of susceptible individuals to an allergen, causing immunological priming—results in stage 2 "the acquisition of skin sensitization". The entire process can take from four days up to three weeks, and during this period of time there may not be any visible signs of skin damage. A sensitized individual can later develop an accelerated secondary response to that very same compound. This means that if and when exposed again to the allergen—at the same location, or somewhere else—an aggressive immune response will be prompted. The skin may start to itch and hurt, turn red, swell and even blister. Generally this inflammation is limited to where the skin came into contact with the allergen, but in very severe cases it may spread and cover large areas all over the body. This reaction usually starts within 12 h of the actual exposure. After three or four days the condition is at its worst. Thereafter, it will slowly (in about seven days) start to heal. This kind of allergic sensitivity may continue to linger, perhaps merely dormant, for the rest of one's lifetime. If the individual does not come into contact with the allergen again, the level of sensitivity may wither gradually.

The chemical components of latex gloves, nickel earrings, and aluminum eyeglasses, to name a few; are well known to be able to cause sensitization by acting as allergens (Fig. 14.1). It is possible for someone who was until recently unaffected by these allergens to suddenly be sensitized. On the other hand, it is also possible for someone to become sensitized to a certain chemical only *after* first exposure to that chemical. For instance, someone who was first sensitized to nickel will later develop skin reactions, like edema and erythema, only a few days after wearing nickel earrings.

14.2 Animal Models

ACD is a dermal cell–mediated type allergic reaction, rather than a humoral or antigen–antibody type. The skin of laboratory animals is used in sensitivity testing. Dermal sensitization reactions in laboratory animals are graded by redness and swelling. Up until 2002, the two most widely used animal models for the evaluation of the skin sensitizing potential of compounds were the guinea pig maximization test (GPMT) (Magnusson and Kligman 1969, 1970) and the occluded patch test of Buehler (Buehler

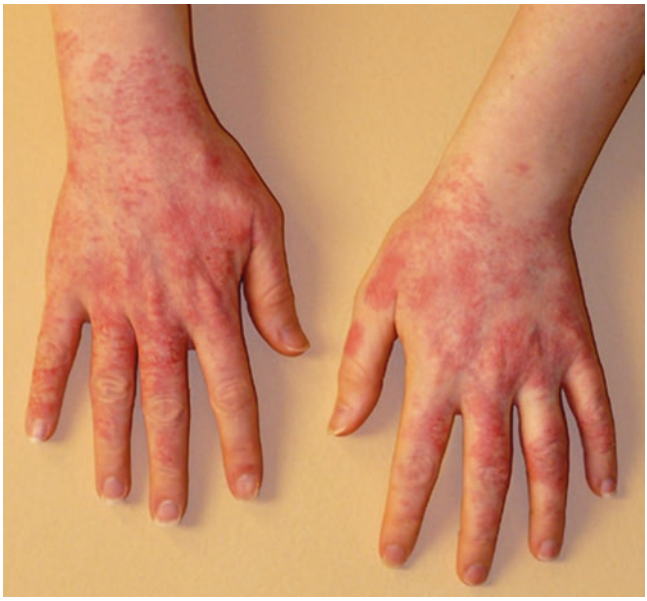

Fig. 14.1 After wearing natural latex gloves daily, a person may develop a rash (http://www.iacd-world.org/skin/latex.htm, 26 February 2016)

1965; Ritz and Buehler 1980). Both models were adopted in the OECD Guidelines for Testing of Chemicals (OECD TG 406) in 1981 (updated in 1992), the US-EPA OPP test guidelines in 1982 (updated in 1996 and 2003) and the Japanese MAFF in 1985. Both models contain the induction phase as well as the challenge phase to elicit a sensitization response. The induction phase consists of an intra-dermal injection and/or epidermal patch application of test compound in test animals, followed by a resting period of 10–14 days, during which an immune response may develop. Control animals are treated in much the same way, except a negative control compound or vehicle is applied rather than the actual test compound. During the challenge phase, both the test and control animals are exposed by a patch to a challenge dose of the test compound applied dermally for 24 h. At several time intervals after removal of the patch, the resulting skin reactions are scored according to the Magnusson and Kligman grading scale (Table 14.1). The extent and degree of skin reactions in the test animals is then compared to the skin reactions of the control animals.

For the GPMT at least ten test animals and five control animals are used. For the Buehler test, a minimum of twenty test animals and five control animals is used. The performance of the GPMT takes approximately 23–25 days. The Buehler test takes approximately 30–32 days. A primary irritation test may be performed in order to select the suitable test compound concentrations for a GPMT or a Buehler study. The concentration of test compound used for each induction exposure should be well-tolerated systemically and should be the highest to cause a mild to moderate skin irritation. For the challenge exposure the highest non-irritant dose should be used. The primary irritation test may use four to eight animals.

Table 14.1 Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions

Grade 0 = no visible change
Grade 1 = discrete or patchy erythema
Grade 2 = moderate and confluent erythema
Grade 3 = intense erythema and swelling

Open source: Organization for Economic Co-operation and Development. OECD Guideline for testing of chemicals (1981) Guideline 406: Skin Sensitisation. OECD, Adopted 12 May 1981, updated 17th July 1992

Although these guinea pig models have been of great value and currently provide the basis for predictive testing, these models have their limitations, not the least of which is the fact that sensitizing potential is determined by subjective assessment of challenge-induced erythema. There was need for a better model which at least provides objective endpoints, and serves as an alternative in terms of animal welfare.

The immune system of the mouse has been investigated more extensively than that of the guinea pig. The mouse ear swelling test (MEST) was developed in the early 1980s to provide lower costs, shorter duration and objectively graded alternative to the existing guinea pig tests (Gad et al. 1986). Over time, the test design has been modified and its use has been extended. The murine local lymph node assay (LLNA) was described by Kimber et al. (1986, 1989; Kimber and Cumberbatch 1992) as a method for assessing the contact sensitization potential of compounds. Since that time, the LLNA has undergone extensive evaluation and validation. In 1999, the validation report (NTP 1999) was published on the website of the US National Institutes of Health (NIH). This makes the LLNA the only validated in vivo method for detection of skin sensitization, and its validation process served as a model for current validation processes.

In July 2002, the OECD adopted the LLNA as stand-alone alternative test and issued OECD TG 429. The EPA's revised test guideline, OPPTS 870.2600 Skin Sensitization, incorporates the LLNA for use as an alternative method for assessing skin sensitization under the appropriate circumstances. The LLNA provides advantages for animal welfare as well as scientific benefits, and since then the majority of guinea pig tests was replaced by the LLNA alternative. In July 2010, an update of OECD TG 429 was published which introduced minor changes to the main protocol and included alternative end points. Currently the LLNA is the preferred assay for the predictive identification of skin-sensitizing chemicals and is the initial requirement for sensitization testing within REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances).

14.3 Delayed Type IV Hypersensitivity Response

ACD is an immune Type IV response—often called *delayed type hypersensitivity*, as it can take up to two or three days for the skin to react. ACD is not antibody-mediated, but a T-lymphocyte (T-cell) mediated allergic reaction.

The induction phase of ACD is triggered after exposure of the individual to a sufficient local concentration of inducing compound. In order to trigger the immune system,

the compound has to pass the first line of defense; the stratum corneum (SC) of the skin. The ability of a compound to pass the SC depends on its reactivity (with high reactivity the penetration will end at SC level), size (compounds larger than 5000 Da will penetrate at insignificantly small levels), polarity and its ability to form hydrogen bounds with proteins. During this passage, the compound may be subjected to biotransformation processes which can both increase or decrease the allergenic potential. After reaching the tissue at the epidermal/dermal level, the compound normally is too small to be recognized by the immune system and has to react with native proteins to form a stable hapten-protein complex, which is large enough to be recognized by the immune system. Following recognition, these complexes are internalized by epidermal dendritic cells (i.e. Langerhans cells) or dermal dendritic cells which form the second line of defense. The dendritic cells mature, migrate out of the dermis and transport the complexes to the draining lymph nodes (DLNs). During migration, the dendritic cells develop into potent immune-stimulatory dendritic cells, which are capable of presenting antigen- containing complexes to naive T-cells in the DLN. This induces differentiation and proliferation of specific T-cells, some of which re-circulate throughout the body (Kimber and Basketter 1992; Kimber and Dearman 1996). In addition during the passage of the compound through the skin, endogenous glycolipids are being released, which are presented by the dendritic cells to natural killer T-cells. These natural killer T-cells release IL-4, which stimulates B lymphocytes to produce IgM. The effective stimulation of a primary immune response is dependent on the allergen reaching the DLNs draining the site of exposure in sufficient quantity (Fig. 14.2).

At this point the individual is sensitized. An accelerated and more aggressive secondary immune response will occur when the trigger allergen is encountered again. After exposure, the hapten-protein complex is formed, and subsequently taken up by dendritic cells. The circulating memory T-cells are triggered to secrete specific cytokines that induce the release of inflammatory cytokines, which mobilizes cytotoxic T-cells and other inflammatory cells from the blood stream. These cells, in their turn, cause a local inflammatory reaction—edema and erythema being ACD's two most characteristic symptoms (Fig. 14.3).

Until recently, the measurement of such cutaneous inflammatory reactions formed the basis of all predictive methods for the identification of skin sensitizing chemicals. For such methods the guinea pig was the species of choice and both the Guinea Pig Maximization Test (GPMT) and Buehler test do comprise both phases: the induction phase and the elicitation (challenge) phase. The skin sensitizing activity is measured as a function of dermal inflammation provoked by the challenge.

Focusing on the induction phase only, the murine local lymph node assay does not have a challenge phase. The contact allergenic potential of a compound is evaluated by its ability to stimulate the cellular proliferation of the DLNs of mice following topical treatment with test compound. This stimulation reflects the induction of an immune response. The strength of this immune response correlates closely to the degree of acquired sensitization. It is this in vivo proliferative response that forms the biological basis of the LLNA as a suitable model for contact sensitization.

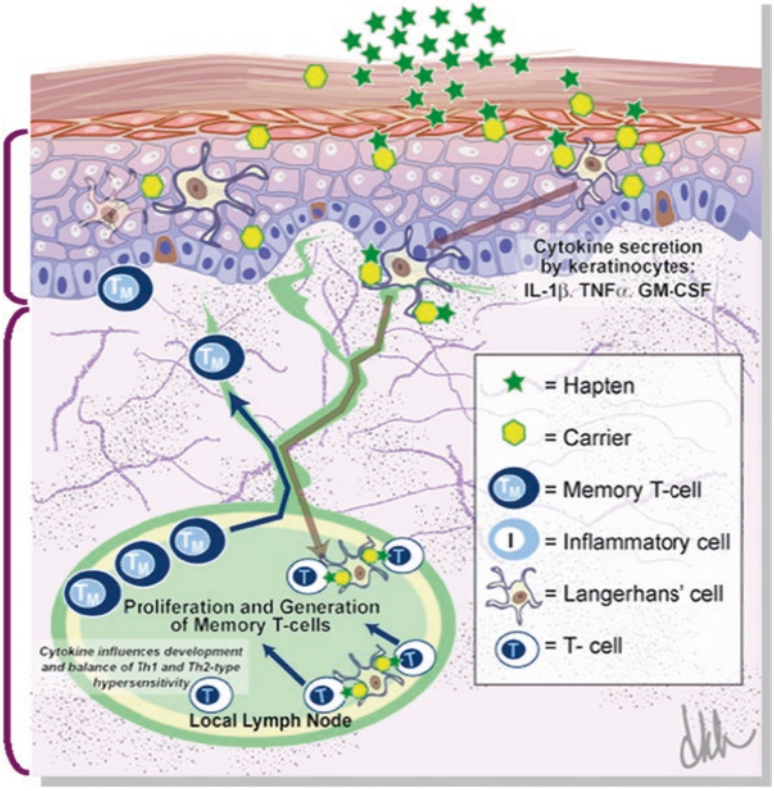

Fig. 14.2 The induction phase of skin sensitization; taken from OECD. (2014), *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins*, OECD Series on Testing and Assessment, No. 168, OECD Publishing, Paris DOI: http://dx.doi.org/10.1787/9789264221444-en. Source: ENV/JM/MONO(2012)10/PART1 OECD environment, health and safety publications series on testing and assessment No. 168

14.4 The Local Lymph Node Assay (LLNA) Principle

Although several variations and methods exist, the general principle of the LLNA is the exposure of mice to test compound on the dorsal side of each pinna (ear flap) and the measurement of the proliferation of the draining auricular lymph nodes. The advantage of using the ears is that the compound is applied on a specific well defined and biologically bordered area, making exposure calculations in terms of dose per square inch/centimeter of skin possible. The skin of the ears is hairless so as opposed to the guinea pig models, there is no need to shave or depilate the skin—actions that might influence the skin's integrity. Using the ears also facilitates the reading of the skin for erythema and the edema formation, and it allows for ear swelling to be easily assessed. The draining lymph nodes of the ears are well located (Fig. 14.4).

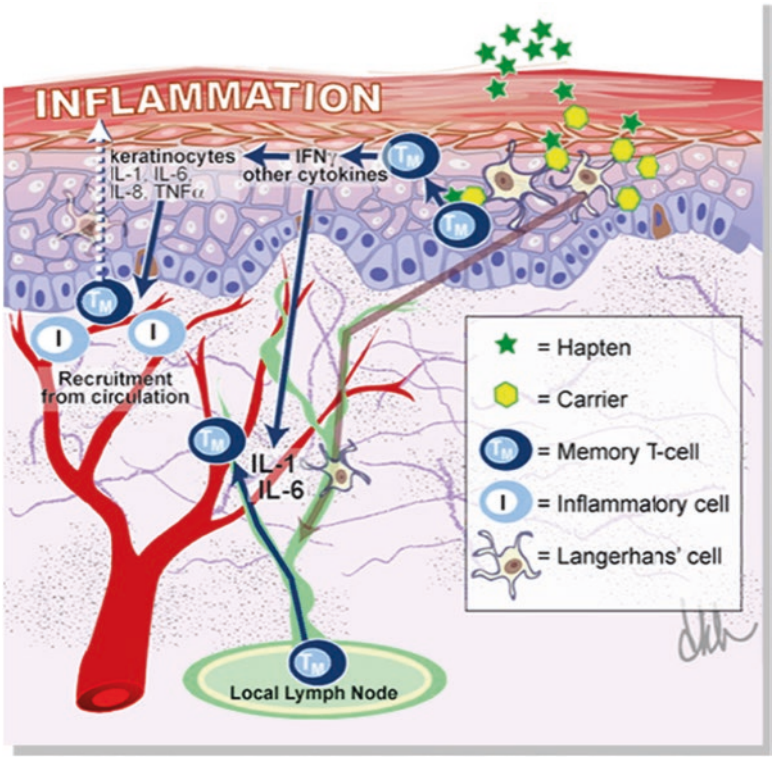

Fig. 14.3 The elicitation phase of skin sensitization; taken from OECD. (2014), *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins*, OECD Series on Testing and Assessment, No. 168, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264221444-en. Source: ENV/JM/MONO(2012)10/PART1 OECD environment, health and safety publications series on testing and assessment No. 168

In the LLNA, a dose volume of 25 μL of test compound is spread evenly over the dorsal side of both ears for three consecutive days. On Day 6, 250 μL of sterile phosphate-buffered saline (PBS), containing tritiated (3H)-methyl thymidine, is injected into the tail vein of all animals. The (3H)-methyl thymidine will be incorporated in all newly synthesized DNA, including the DNA that is synthesized during the proliferation of the cells in the DLNs. After 5 h, the DLNs of the ears are harvested and collected in cold PBS buffer. Lymph Node Cell suspensions are prepared by gentle mechanical disaggregation, and the cells are washed and resuspended in trichloroacetic acid for at least 18 h at 4 °C in order to precipitate the DNA. The incorporation by local lymph node cells of tritium labeled thymidine is measured by β-scintillation counting. The results are expressed as mean disintegrations per minute (DPM) for each group (Fig. 14.5).

The typical LLNA consists of three test groups; each group consisting of five mice, each group tested with one concentration of test compound, and one concur-

rent control group dosed with vehicle. In addition, one concurrent positive control group may be added. The proliferation of the DLNs can be measured pool-wise for each group, or individually by pooling the left and right node for each animal. Both for scientific as well as for animal welfare reasons, the individual approach is preferred, since it offers the possibility to apply statistical analyses, assess inter-animal variability and elimination of possible outliers. Using the group's mean DPM values, the ratio between the mean DPM values for each test group to the mean DPM value of the concurrent vehicle control group is calculated. This ratio is called the Stimulation Index (SI). By using concurrent control groups any day-to-day variation is taken into account. Those compounds that at one or more concentrations are able to induce an SI = 3 are considered to be contact allergens.

In contrast to the guinea pig tests which are performed at a single concentration, the LLNA utilizes three concentrations allowing for the assessment of a dose response relation. The dose response relation provides a more complete evaluation of the test result and the use of three concentrations enables statistical analysis based on individual animal data. Moreover, it also enables the determination of the potency of test compounds. This potency is reflected in the EC3 value (the estimated concentration needed to produce a stimulation index of 3). Although several methods exist to determine the EC3 value, the most commonly used method is the calculation by simple linear interpolation using the data points of the dose response curve that lie immediately above and below the SI = 3 value (Basketter et al. 1999). If these data points have the coordinates (a, b) and (c, d) respectively, then the EC3

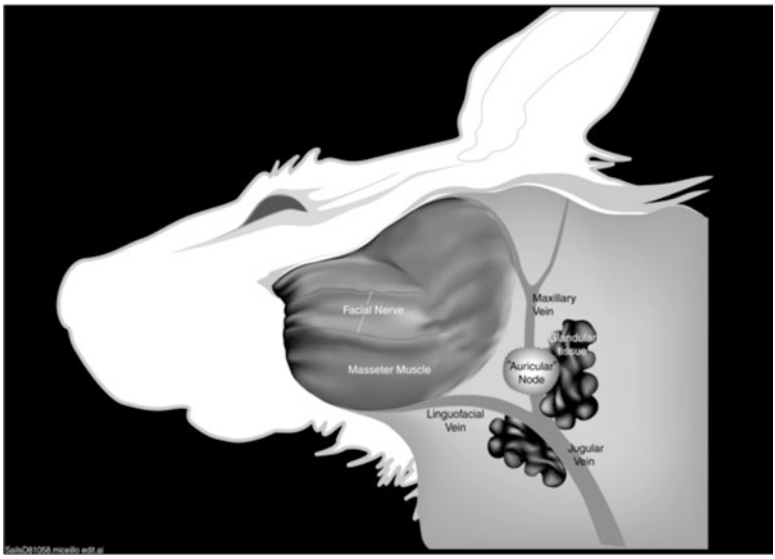

Fig. 14.4 The location of the draining lymph nodes of the ears of the mouse. Taken from: ICCVAM. 2009. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication Number 09-7357. Research Triangle Park, NC: National Institute of Environmental Health Sciences

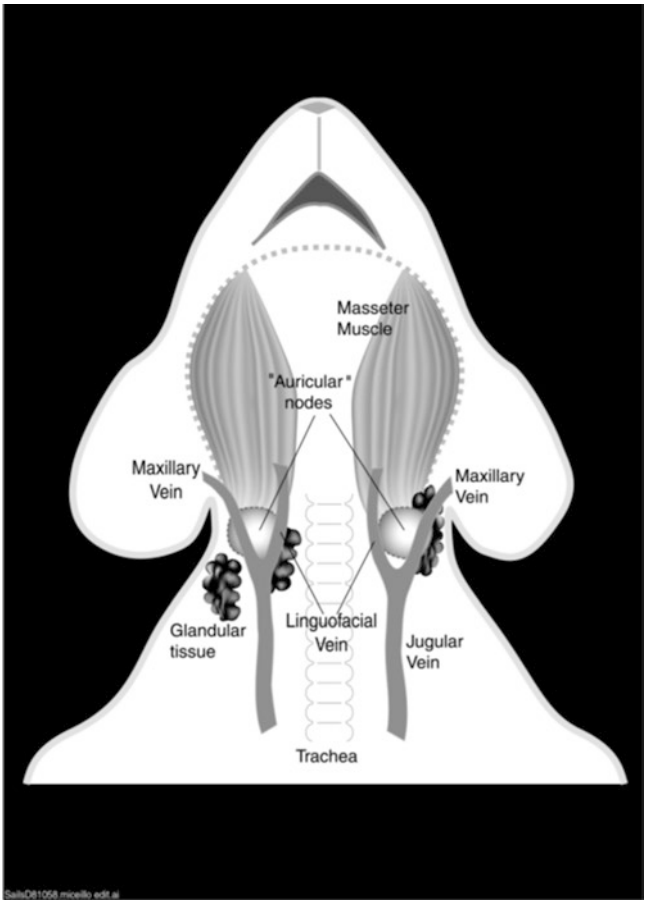

Fig. 14.4 (continued)

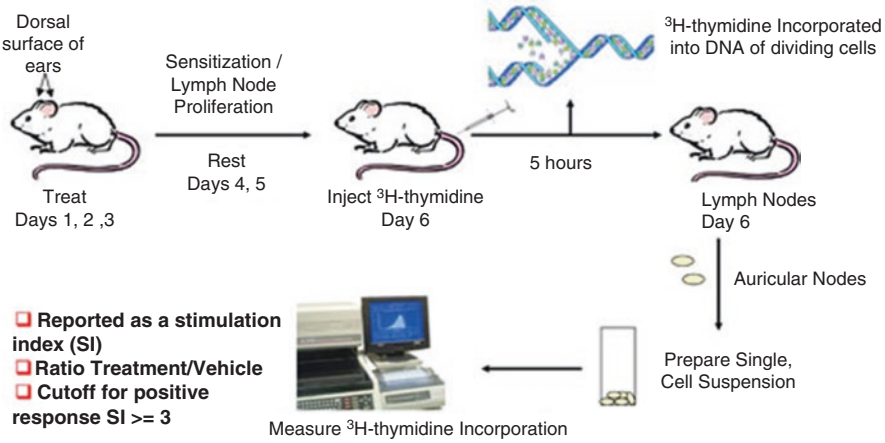

Fig. 14.5 The LLNA outline (Retrieved from: http://www.specchemonline.com/articles/view/on-the-right-track#.VcRh3rXSrcs On 07-Aug-2015)

value may be calculated using the equation: EC3 = c + [(3 − d)/(b − d)](a − c)]. The theoretical 0,0 point is not considered to be an actual data point and therefore cannot be used within this method. When no data points appear below the SI = 3 value, a more complex log-linear extrapolation may be applied, in which the two lowest test concentrations from the dose response curve are used (Fig. 14.6).

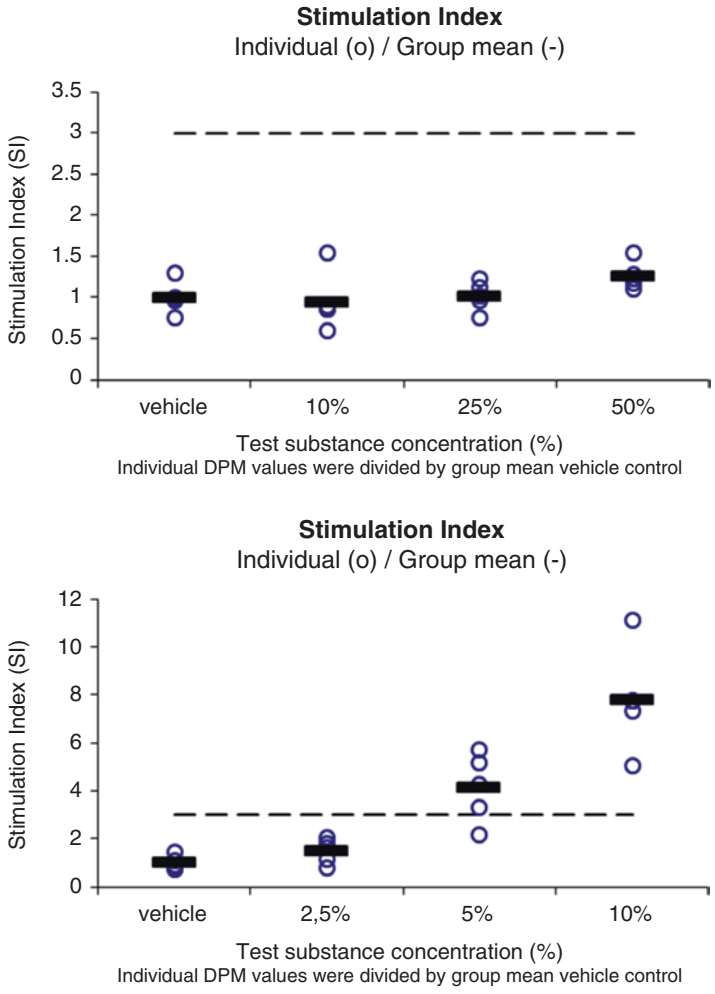

Fig. 14.6 Examples of LLNA study results. Top figure shows an example of a negative response. Three groups of five mice were dosed topically on the ears with test substance concentrations of 10, 25 and 50%, respectively. One group was dosed with vehicle. There was no response to the dose and none of the test compound concentrations exceed the SI = 3 threshold. The lower figure shows an example of a positive response. Three groups of five mice were dosed topically on the ears with test substance concentrations of 2.5, 5 and 10%, respectively. One group was dosed with vehicle. There was a test substance related response to the dose with SI values exceeding the SI = 3 threshold. An EC3 value of 3.9% was calculated using linear interpolation

14.5 Alternative Methods for the Assessment of Cell Proliferation

The traditional OECD TG 429 LLNA requires injection of radioactive material. However, since there are not many labs that are sufficiently equipped for working with radioactive materials, several non-radioactive methods have been developed to determine the cell proliferation. Two of these methods were adopted in the OECD test guidelines in 2010. The LLNA:DA method (OECD TG 442A) measures cell proliferation by assessing the level of ATP using a bioluminescence assay. The LLNA:BrdU-ELISA method (OECD TG 442B) measures cell proliferation by assessing the level of incorporation of BrdU into newly formed DNA by ELISA testing.

Another successfully validated alternative method utilizes the cell counts of the lymph nodes using a Coulter Counter as well as the lymph node weights to assess cell proliferation rather than radioactively labeling them (Ehling et al. 2005a, b; Kolle et al. 2012; Ulrich et al. 2001; Vohr et al. 2000). In addition, the acute inflammatory skin reaction is measured by ear swelling that is reflected in the weight of circular biopsies of the ears. Similar to the EC3 values defined for the radioactive method, threshold concentrations are calculated for the endpoints measured in this modified LLNA. This method also shows that all measured parameters have to be taken into consideration for the final assessment of whether or not the compound acts as an allergen. Therefore, an assessment system was developed. This system turned out to be of great importance to consistently assess sensitization versus irritancy, based on the data of the different parameters. For example, an irritant compound may result in pronounced edema formation and cellular infiltration, therefore, the skin of the ear is substantially thickened, but the pathologic process lacks the degree of local cellular proliferation that characterizes a true sensitivity reaction. As opposed to applying the radioactive method, irritants can be characterized more accurately using this assessment system (Table 14.2).

Ex vivo incorporation of labeled thymidine is another possibility to determine cell proliferation which reduces the amount of radioactive waste and disposal of

Table 14.2 Assessment scheme for the modified LLNA results

Assessment of results								
Endpoint result								
Lymph node weight	+	+	+	+	–	–	–	–
Lymph node cell counts	+	+	–	–	+	+	–	–
Assessment I	A/I	A/I	I	I	A	A	–	–
Acute skin reaction[a]	–	+	+	–	–	+	+	–
Assessment II	–	I	I	–	–	I	I	–
Overall assessment	A	A/I	I	I ?	A	A/I	I	–

Source: Ehling et al. (2005b) An european interlaboratory validation of alternative endpoints of the murine llna second round. Toxicology 212:69–79

A, Allergen; *I*, irritant; *A/I*, allergen with pronounced irritating potential or strong irritant

[a]Measured by ear swelling or ear weight

radioisotope-contaminated carcasses (Piccotti et al. 2006). The single cell suspensions of the lymph nodes are placed in culture medium containing tritiated (3H)-methyl thymidine and cultured for 24 h. The incorporation of tritium-labelled thymidine by local lymph node cells is measured by β-scintillation counting.

14.6 Critical Performance Factors

Several factors should be taken into account when designing an LLNA study. It is essential for the vehicle to be selected carefully to insure that it will not interfere with the test result or bias its outcome. There are cases in which the vehicle did to some extent affect the outcome of the assay. The most extensively used vehicle is a mixture of four parts of acetone and one part of olive oil (4:1). Other vehicles commonly used are *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, dimethyl sulphoxide, *N,N*-dimethylacetamid/acetone/ethanol (4:3:3) (DAE433) and ethanol/water (7/3, v/v). When the clinical formulation—or marketed formulation—is tested, it may be necessary to include the relevant solvent as an additional control in order to assess the effects of the solvent on the test system. It is preferred to test solutions instead of suspensions in order to maximize penetration into the skin. Using solutions optimizes the dispersal over the ears surface and reduces possible contamination of the animal's environment and oral uptake of test compound that has inadvertently remained on the surface of the ear. However, reliable results can also be obtained by applying suspensions and highly viscous compounds. In the case of hydrophilic compounds, particular care should be taken; these compounds can run down and fall off the ear due to the hydrophobic environment of the skin. Compounds such as these can be incorporated into a vehicle that merely wets or moistens the skin and therefore preventing the test compound from immediately running off the test site. An appropriate solubilizer such as 1% Pluronic® L92 (Ryan et al. 2002), or hydroxyethyl cellulose can be added to help hold the solution on the ear until it is fully absorbed. Defatting the skin of the ears with acetone immediately prior to dosing can be used to help facilitate the spreading of an aqueous solution over the surface of the ear, and thereby enhancing the absorption of the compound.

It is important that the applied compound concentrations are systemically well-tolerated by the animals. Since significant effects on parameters such as body weights and clinical signs may influence the sensitivity and response of the animals, it is important to monitor these parameters very carefully. Distinctive criteria to which extent systemic effects are acceptable do not exist. However, when testing multiple concentrations the dose response relationship can aid in detecting effects on the sensitivity of the assay at higher concentrations.

Non-specific stimulation of the lymph nodes by irritation of the ears is of great concern. In contrast to guinea pig studies, where the induction and challenge phase are well separated in terms of time and dose location, the dose location in the LLNA study (namely the ears) and the draining lymph node (as endpoint marker) are located closely together, and the response is measured within only a few days after

Table 14.3 Grading of erythema for the ears of the mice

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

Open source: Organization for Economic Co-operation and Development (OECD) (2010) OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.429, "Skin Sensitization: Local Lymph Node Assay", Paris Cedex, July 2010

exposure. Disturbances such as irritation at the skin site may directly influence the lymph nodes. Although the great majority of non-sensitizing irritants are negative in the LLNA, chemically induced cellular proliferation has been observed with some irritants. This proliferation has been thought to be nonspecific and non-antigen directed (Gerberick 1992; Montelius et al. 1994). It is therefore important for the local effects that are elicited by the applied concentrations on the ears to be monitored carefully for any signs of irritation. There are multiple ways of assessing these signs: visible examination of the ears (for erythema and edema), measurement of the ear thickness (to determine the ear swelling) or a post-mortem weighing of the ear biopsies. "Excessive irritation should be avoided," states OECD TG 429. When scoring the ears for erythema according to the table below (Table 14.3), excessive irritation is defined as 'erythema scores 3' or 'erythema score 4'.

Also, when—at any time during the study—an increase in ear thickness exceeds 25% (in comparison to pre-dose values), or an increase in ear biopsy weights exceeds 25% (in comparison to the control animals) this is to be considered excessive irritation. By applying these criteria, one can avoid interpreting false positive outcomes that are actually caused by irritation.

When no information regarding the mentioned critical factors is available, a pre-screen test can be performed. This will allow for an investigation of whether or not the compound concentrations to be tested in the LLNA main study are suitable. The pre-screen test is conducted under conditions identical to the main LLNA study. However, there may be no assessment of lymph node proliferation and it possible for fewer animals per dose group to be used.

14.7 LLNA Negative and Positive Control Data

The LLNA proved to be a robust test system. However it is important to have historical control data available in order to demonstrate the appropriate performance of each individual assay. Each assay must contain a concurrent vehicle control group for comparison and determination of the SI. The reliability of the response of the vehicle control group is crucial since it directly affects the magnitude of the SI's calculated for the test groups. The results obtained for individual vehicle control

groups should be compared with a vehicle control data base in order to assess the reliability of the result obtained for the individual assay. The vehicle control data base should show consistency over time.

To demonstrate appropriate performance of the assay, positive controls are used. These controls should respond with adequate and reproducible sensitivity to known sensitizers for which the magnitude of the response is well-characterized. Most widely used positive control substances are known sensitizers like hexyl cinnamic-aldehyde, mercaptobenzothiazole and 2,4-Dinitrochlorobenzene. Positive controls can be added as a separate group to each assay, but it is also possible to periodically perform a single assay with them. Inclusion of a concurrent positive control to each assay allows for an assessment of intra-, and inter-laboratory reproducibility and comparability. However, it might be necessary to include an additional concurrent vehicle control to the assay for comparison with the positive control if the vehicle used for the test compound differs from the vehicle used for the preparation of the positive control. For laboratories that perform the LLNA at a regular basis, periodic performance of a positive control assay (i.e. at intervals = 6 months) may suffice. The advantage is that a periodically performed assay with three concentrations allows for the inclusion of the dose response relation assessment in the evaluation, as well as the determination of the EC3 value, to determine the sensitivity of the assay. Apart from these scientific advantages, the use of a periodic positive control will also reduce the number of animals needed. The disadvantage of a periodic positive control, however, arises in case of negative results. In that case, all negative individual LLNA's performed earlier on should be questioned.

14.8 Limitations to the LLNA

As any animal model, the LLNA does have its limitations. For instance, irritants should be tested with care, since it is known that excessive irritation of the ears may lead to false positive results. Also, strong irritants may trigger cytokine production at the site of irritation, resulting in an increase in lymph node cellular proliferation that is not due to hypersensitivity. It has been shown that interleukin 1α—released in case of irritation—was able to induce the migration of Langerhans cells to the draining lymph nodes. The arriving cells showed phenotypic characteristics of dendritic cells, which are similar to cells that are activated in the skin when a skin sensitizing chemical is applied topically. In order to avoid non-specific stimulation of the nodes, an accurate selection of the suitable (highest) concentrations of test compound to be tested in the LLNA should be made. For this purpose, the appropriate irritation endpoints and selection criteria are to be used. The irritating properties of a compound may form a limitation with regards to the maximum concentration that can be tested—and as such may reduce the strength and relevance of the assay. The same limitation accounts for the challenge in a guinea pig study which should be performed at non-irritating levels, thus also limiting the maximum concentration that can be tested.

In order to distinguish between irritant and allergic responses in the LLNA, several modifications and alternative endpoints for the LLNA have been investigated. One of these modifications uses flow cytometry in order to measure specific B-cells (Gerberick et al. 1999). Although contact sensitization is considered a T cell mediated immune response, mice exposed to contact allergens do show an increase in B-cells in stimulated lymph nodes. Specifically, selective modulation of B-cells carrying a specific marker, B220+ cells, was observed after exposure to allergen and not to irritant.

Another limitation of the LLNA may be the technical applicability of test compound to the ears. In contrast to the guinea pig studies, in which the test compound is injected or kept in place using dressings and bandages, in the LLNA the test compound is dosed on the ears without any further covering of the dosed location. The consequence is that highly viscous compounds, or compounds with very low solubility, cannot be dosed properly—as they might simply fall off the ears. Another risk (though considered small and negligible) is the ingestion of the compound by the animals after it starts grooming itself or its cage mates. The amounts of compound ingested is normally too low to have any relevant effect on the animals. Nonetheless, it can be concluded that, for an optimal LLNA performance, it is recommendable to use solutions that are quickly absorbed by the skin.

In time, experience with the LLNA revealed that in many cases surfactant type chemicals showed false positive findings, regardless of their irritating properties. For these types of compounds the guinea pig assays are currently considered to provide a better predictability of the sensitizing properties (Basketter et al. 2009; Garcia et al. 2010).

Despite its limitations, the LLNA is deemed appropriate for testing most types of compounds. At least the limitations are well-known, which cannot be said for other in vivo studies—as these have not undergone validation. A key perspective is that no predictive test is without its limitations, and having a good appreciation of these limitations is necessary when putting the information derived from any method to best use.

14.9 Advantages to the LLNA

Compared to the guinea pig tests, the LLNA is an improved method regarding to animal welfare, but is an in vivo method nonetheless. The use of animals cannot completely be abolished, however the number of animals used is reduced. Moreover, the LLNA offers a substantial refinement (less pain, less distress) in the manner in which animals are being used for allergic contact sensitization testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitization. Unlike guinea pig tests, the LLNA does not require the elicitation of challenge-induced dermal hypersensitivity reactions. It is therefore also shorter in duration, since the assay takes only 6 days—as opposed to the minimum of 24 days of a guinea pig test. Furthermore, the LLNA does not require the use of an adjuvant, as opposed to the guinea pig maximization test, and

it does not involve injections or bandages to apply the test compound. Thus, the LLNA reduces animal discomfort.

There are also several scientific advantages. The LLNA is the first fully validated test method and the protocol has been optimized for best performance. Compared to the guinea pig studies, the LLNA provides objective and quantitative endpoints and is less prone to subjective interpretation. It also provides a dose response assessment which in itself provides important information on the relative potency of a potential skin sensitizer as well as the possibility to categorize and classify sensitizers from mild to severe. This is of great importance for risk assessment as well as a proper safety evaluation.

Another advantage is that he LLNA is also used to identify respiratory allergens. It has been shown that contact allergens preferentially induce a T-helper 1 (TH1) response, whereas respiratory allergens preferentially induce a T-helper 2 (TH2) response. These responses can be discriminated on the basis of cytokine production, such as IFN-gamma, which is produced by TH1 cells, and IL-4, which is produced by TH2 cells (De Jong et al. 2009). A practical tiered approach for testing of respiratory allergens has been described based on the observation that most if not all known respiratory allergens tested, have also tested positive in this assay (Arts and Kuper 2007). This suggests that a chemical which fails to induce a positive response in the LLNA most probably lacks the potential for respiratory allergy.

14.10 Non-Animal Models

Integrated testing strategies (ITS) composed of (Q)SAR, and molecular and/or cell-based assays are being validated to form predictive test batteries to identify skin sensitizers and replace animal testing (Adler et al. 2011). Although the immune response is a complex event of several steps—breaking down the process in skin penetration, binding to proteins and reactivity—it did help develop in silico, in chemico and in vitro methods. Three alternative methods that are of interest are the in chemico Direct Peptide Reactivity Assay (DPRA), and the cell based KeratinoSens™, and the human Cell Line Activation Test (hCLAT).

The DPRA is a test method that provides information on peptide reactivity by measuring the depletion of two synthetic peptides, which is considered to be the molecular initiating event of skin sensitization.

The KeratinoSens™ is a test method that provides information on the ability of a chemical to activate the Nrf2 electrophilic and oxidative-stress response signaling pathway, which has been shown to be a relevant pathway in the induction of skin sensitization (i.e. activation of keratinocytes).

The human Cell Line Activation Test (h-CLAT) quantifies the induction of protein markers, associated with dendritic cell maturation in vivo, on the surface of dendritic cell-like cell lines following exposure to the chemical.

These three assays represent biological mechanisms covered by the OECD Guidance Document (GD) on Developing and Assessing Adverse Outcome Pathways (AOPs) (OECD GD 184, 2013).

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Chapter 15
Host Resistance Assays for Efficacy and Immunotoxicology Safety Evaluations

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Abstract The purpose of immunotoxicity testing is to obtain data that is meaningful for safety assessment and a number of approaches are available to achieve this goal. Regardless of the approach, it is important that the testing evaluate the three arms of the immune system and include assessments of innate immunity, CMI, and HMI.

When host resistance is used for immunotoxicity testing, immune suppression caused by a test compound or chemical may be reflected as impaired clearance of the infectious agent, increased sensitivity to opportunistic infections, prevention of immunization, or exacerbation of latent infections. In this immunotoxicity testing paradigm, the host resistance assay allows for an assessment of total functional immunocompetence and serves as a biomarker of net immunological health and well-being.

It is difficult to assess the biological relevance of an 'x' percent change in a particular immune parameter, such as reduction in thymus weight or reduction of NK activity, unless this change is evaluated in terms of overall biological impact. Host resistance assays, with the available redundancy inherent in the immune system, allow such an evaluation.

Comprehensive host resistance assays are used to evaluate the overall health of the immune system, i.e., how the numerous components of the functional immune system work together to clear an infection, while targeted host resistance assays are available to evaluate specific immunotoxicity questions. The choice of which host resistance assay to employ is dependent on the question being asked, and on the context of the question being posed (immunotoxicity or efficacy).

Keywords Immunotoxicity testing • Functional assays • Innate immunity • Cell-mediated immunity (CMI) • Humoral-mediated immunity (HMI) • IgM antibody • IgG antibody • Marginal zone B cells (MZB cells)

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15.1 Approaches to Evaluate Immunotoxicity

The purpose of immunotoxicity testing is to obtain data that is meaningful for safety assessment, the primary objective of which is to determine the significance of an immunotoxic effect with respect to immune suppression and increased susceptibility to infectious or neoplastic disease, or immune enhancement and hypersensitivity or autoimmunity.

Host resistance assays and animals models of disease are used as the principal method in evaluating the efficacy of antibacterial, antiviral and antifungal compounds during drug development. When host resistance is used for immunotoxicity testing, immune suppression caused by a test compound or chemical may be reflected as an impaired clearance of the infectious agent, increased sensitivity to opportunistic infections, prevention of immunization, or exacerbation of latent infections. In this paradigm, clearance of an infectious microorganism in a host resistance assay allows for an assessment of total functional immunocompetence and serves as a biomarker of net immunological health and well-being. The choice of which host resistance assay to employ is dependent on the question being asked, and also on the context of the question being posed (immunotoxicity or efficacy).

There are a number of approaches to evaluate immunotoxicity. A tiered approach is commonly used that evaluates standard toxicity tests followed by immunological function and host resistance.

15.1.1 Standard Toxicity Studies (STS)

Standard toxicity studies include hematology, clinical chemistry, gross pathology, organ weights and histology. The STS screening assays provide some initial information on the immune cells and organs that are effected by drug treatment or chemical exposure.

15.1.2 Functional Assays

Functional assays extend the findings in the STS screening assays and help determine if the STS effect on immune cells and organs translates into a defect in immune function. Functional assays should include a thorough evaluation of the three arms of the immune system: innate immunity, cell-mediated immunity (CMI), and humoral-mediated immunity (HMI).

Innate immunity, a nonspecific immune response involving immunological mediators that recruit and activate innate immune cells, is considered the first line of immune defense following immune challenge. The cytokine and chemokine

immunological mediators serve an important role in cell–cell communication among cells of the immune system, and call-in and activate immune system cells. The main cell types recruited and activated in the innate response include natural killer (NK) cells, macrophages, and neutrophils, among others. By virtue of their location throughout the body and their specialized functions, these cells facilitate a critical early interaction and are important contributors to early nonspecific innate immunity, and participate in specific immunological responses as well. Several assays exist to examine innate immune function. The measurement of NK cell activity, for example, is an important functional assay of innate immunity.

CMI is an adaptive immune response that involves antigen-specific recognition by T cells. Delayed type hypersensitivity (DTH) and cytotoxic T lymphocyte activity (CTL) are used to assess CMI in vivo. The CTL response requires the function and interaction of several categories of immune cells, including professional antigen-presenting cells such as dendritic cells and/or macrophages, $CD4^+$ T lymphocytes that produce help for response to T-dependent antigens, and $CD8^+$ T lymphocytes that develop into antigen-specific cytotoxic effector cells. In the context of host resistance, antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL) mediate the cell killing of intracellular pathogens or tumor cells (Burleson et al. 2010).

HMI is an adaptive immune response characterized by the production of antibodies including IgM and IgG. The antibody produced is important in preventing infection by infectious microorganisms, if previously immunized, or can be an important immunological therapeutic in limiting spread and eliminating infectious agents. The antibody response may be classified as a T-dependent antibody response (TDAR) or as a T-independent antibody response (TIAR).

The TDAR response requires and measures the functionality of three major immune cell types: T cells, B cells, and antigen presenting cells such as dendritic cells and macrophages. Measurement of TDAR is important in assessing the ability of the host to produce antibody. TDAR may be measured by evaluating the number of antibody forming cells (AFC) in the spleen following immunization with sheep red blood cells (SRBC). The SRBC AFC assay uses a technique of Jerne and Nordin (Jerne and Nordin 1963). TDAR may also be measured by immunizing with T dependent antigens such as keyhole limpet hemocyanin (KLH), tetanus toxoid, hepatitis B surface antigen, or SRBC and measuring the antigen specific antibody in the serum by ELISA. The ELISA method measures antibody in the serum from immunized animals and therefore results in a systemic evaluation of antibody production, rather than only in the spleen for the AFC method (White et al. 2007). Antibody class switching can also be evaluated in the serum if analysis includes both the IgM and the IgG antibody response.

The TIAR is important in recognizing polysaccharide antigens, such as those present on the encapsulated bacteria that cause bacterial pneumonia. This antibody response occurs in the absence of T cell help and requires the presence of marginal zone B cells (MZB cells) (Burleson and Burleson 2008a, b).

15.1.3 Host Resistance Assays (Animal Disease Models): The "Gold Standard"

Luster initiated a series of seminal studies that form the basis of risk assessment in immunotoxicology evaluations (Luster et al. 1988; Luster, Pait, et al. 1992; Luster, Portier, et al. 1992; Luster et al. 1995). These seminal studies evaluated various immunological assays that predicted immunotoxicity, and reported concordance values if the test material resulted in a dose-dependent response or altered two or more immune assays, as compared to host resistance assays. Since the primary responsibility of the immune system is to protect against infectious or neoplastic disease, host resistance assays are considered to be the ultimate predictor of adverse effects (Germolec 2004), and are the gold standard for immunotoxicological evaluation.

Many immunotoxicologists regard host resistance assays to be the most relevant for both validating the usefulness of other detection methods and for extrapolating the potential of a substance, drug or chemical, to alter host susceptibility in the human population (Germolec 2004). In general, host resistance assays determine if a drug or chemical results in an adverse effect (decreased immunity to the infectious agent), and provide potential mechanism(s) underlying the adverse effect (alterations related to cytokines, innate immune function, or adaptive immunity).

While mortality host resistance studies have been used more widely in the past, evaluation of immunity in host resistance models where mortality is not an endpoint is more consistent with current animal welfare concerns and the 3Rs. Challenging the immune system with an extremely virulent or with an extremely high titer of the infectious agent overwhelms the immune system with death occurring before development of the cascade of immunological responses required for clearance of the infectious challenge organism. Challenge in excess may reflect a model of sepsis or "cytokine storm" rather than a method to evaluate immunotoxicity. Immunological clearance of the infectious challenge agent is a more sensitive and meaningful measure of immunological function (Burleson 1995; Selgrade and Daniels 1995; Lebrec and Burleson 1994) than mortality. To evaluate bacterial clearance, the number of infectious particles per organ or per gram of organ is quantified at various time points following administration of the test article or chemical.

Immunological reserve can only be evaluated using host resistance assays. The immune response to an infectious agent results in the production of cytokines and chemokines, activation of neutrophils, macrophages and NK cells (innate immunity), activation of cell-mediated immunity (CMI), and lastly activation of humoral-mediated immunity (HMI). If, for example, there is some suppression of NK cell function, the functions of macrophages, neutrophils, CMI and HMI may be able to successfully combat the infection. The remaining functional components of the immune response thus function as the immunological reserve required to successfully combat the infection and can only be evaluated by use of host resistance assays.

With regard to the available models, the emphasis should not be placed on the particular viral, bacterial, or fungal agent that serves as the antigenic challenge, but rather on how the immune system responds to a challenge with a natural antigen.

Host resistance assays provide the only means to directly assess the functional reserve of the immune system, and may be classified as comprehensive host resistance assays or targeted host resistance assays. Comprehensive host resistance assays are used to evaluate the overall health of the immune system, i.e., how the numerous components of the functional immune system work together to clear an infection, while targeted host resistance assays are available to evaluate specific immunotoxicity questions.

15.2 Comprehensive Host Resistance Assay

15.2.1 Influenza Virus Host Resistance Assay

The influenza model in mice or rats is used to evaluate the overall health of the immune system (Table 15.1). Influenza virus host resistance models in mice and rats do not cause mortality, and are perhaps the most thoroughly characterized of all host resistance models. The clearance of influenza virus requires an intact and functional immune system that incorporates a cascade of immune responses and provides a measure of the health of the immune system. Mechanistic immune functions may be measured alongside viral clearance, and may include cytokine levels, macrophage activity, NK cell activity, CTL activity, and influenza-specific IgM and IgG. Measurement of these immunological functions provides an evaluation of innate immunity (cytokine, macrophage, and NK activity), an evaluation of CMI (CTL activity), and an evaluation of HMI (influenza-specific IgM or IgG). Additionally, measurement of influenza-specific IgM or IgG provides a measurement of the TDAR as influenza is a T-dependent antigen (Burleson 1995; Lebrec et al. 1996; Burleson 2000; Burleson and Burleson 2007, 2008a, b).

Table 15.1 Comprehensive host resistance assay

Influenza virus host resistance model
Evaluation of viral clearance as a measure of the integrated immune response
Additional mechanistic endpoints evaluated in this host resistance assay may include:
• *Histopathology*
• *Immunophenotyping and expression of surface markers on immune cells*
• *Cytokines and chemokines—immune mediators*
• *Macrophage activity—innate immunity*
• *NK cell activity—innate immunity*
• *CTL activity—cell-mediated immunity*
• *Influenza-specific antibody (IgM and IgG)—humoral-mediated immunity (TDAR)*

15.3 Targeted Host Resistance Assays

While the influenza host resistance assay is used to assess the overall health of the immune system, targeted host resistance models are available to answer specific questions concerning the nature of a potential immune deficiency (Table 15.2). Targeted host resistance assays may be used if a deficit has been identified, or if a deficit is of concern based on screening assays, functional assays, or data related to test articles with a similar chemical structure or mechanism of action. Targeted host resistance assays determine whether the specific immune deficit in question is adverse, i.e., does a percent decrease in a particular immunological parameter translate to a decreased clearance of the infectious agent.

15.3.1 Bacterial Host Resistance Assays

15.3.1.1 Evaluation of Neutrophil and/or Macrophage Function

Streptococcus pneumoniae Host Resistance Model

Rodent models for bacterial pneumonia have been used to evaluate the potential for immunotoxicity resulting from therapeutics targeting neutrophil and macrophage function. Macrophages were demonstrated to be important in the clearance of streptococci from the lungs of mice (Gilmour et al. 1993) and rats (Gilmour and Selgrade 1993). Further studies by Gilmour and Selgrade demonstrated the importance of neutrophils in pulmonary streptococcal disease in rats by pretreatment with an antibody to neutrophils. The *Streptococcus pneumoniae* host resistance model in rodents has been used in numerous immunotoxicity evaluations in BALB/c and C57BL/6 mice, as well as Fischer 344 (CDF), Lewis, and Sprague Dawley (CD) rats (Steele et al. 2005). In this model, rodents are infected intranasally, and bacterial clearance is measured and compared between test and control groups within 48 h, before the development of adaptive immune responses (Gilmour and Selgrade 1993; Gilmour et al. 1993; Burleson and Burleson 2006). Bacterial clearance is determined by

Table 15.2 Targeted host resistance assays

Host resistance assays for evaluation of specific immunotoxicity endpoints	
Evaluation of defect in neutrophil and/or macrophage function	*Streptococcus pneumoniae* pulmonary host resistance model *Pseudomonas aeruginosa* pulmonary host resistance model *Listeria monocytogenes* systemic host resistance model
Evaluation of immune effect of anti-inflammatory therapeutics	*Streptococcus pneumoniae* pulmonary host resistance model
Evaluation of immune effect of therapeutics targeting TNFα	*Streptococcus pneumoniae* pulmonary host resistance model
Evaluation of defect in Marginal Zone B (MZB) cell	*Evaluation of TIAR*—Systemic *Streptococcus pneumoniae* host resistance model to evaluate MZB cells

determining the number of colony forming units (CFU) per gram of lung tissue. Dexamethasone or cyclophosphamide is generally used as a positive immunomodulatory control as they have well-documented immunosuppressive effects on innate immunity and bacterial clearance. Cytokines may also be measured in the streptococcal model. Cytokines may be measured in the lung as well as in the serum. Bacterial titers and bacterial clearance are quantified as the number of colony forming units (CFU) per organ or per gram of tissue. Macrophage and/or neutrophil function assays may be measured as a mechanistic probe if an effect on bacterial clearance is observed. However, the conclusive observation is bacterial clearance. The *S. pneumoniae* host resistance model has been used in numerous therapeutic evaluations to evaluate the potential for pharmaceutical agents to induce neutrophil and/or macrophage immunotoxicity, and was reported as one of a battery of three host resistance assays to evaluate a small molecule therapeutic targeted for splenic tyrosine kinase (Syk) in mice (Zhu et al. 2007).

Pseudomonas aeruginosa Host Resistance Model

Pseudomonas aeruginosa is used as a pulmonary bacterial host resistance model to evaluate the immunotoxicity of therapeutics when an immunotoxic effect is suspected in neutrophils and macrophages (Gosselin et al. 1995). *Pseudomonas aeruginosa* a Gram negative bacillus that is a human pathogen that primarily causes diseases of the urinary tract, burn patients, septicemia, abscesses, corneal infections, meningitis, bronchopneumonia, and subacute bacterial endocarditis. Treatment often fails and the mortality rate in Pseudomonas septicemia has been reported to be greater than 80%. As in the *S. pneumoniae* pulmonary model, rodents are infected intranasally, and bacterial clearance is measured and compared between test and control groups within 48 h, before the development of adaptive immune responses. Bacterial clearance is determined by determining the number of CFU per gram of lung tissue.

Listeria monocytogenes Host Resistance Model

The *Listeria monocytogenes* host resistance model is a systemic infection assay that is used in the evaluation of adverse effects on neutrophils and Kupfer cells of the liver as well as splenic macrophages and neutrophils. NK cells and T lymphocytes also play a role in bacterial clearance. This host resistance model has been used to evaluate monoclonal antibodies directed against CD11b/CD18 (Mac-1), a leukocyte integrin that plays an important role in neutrophil adhesion and the acute inflammatory process and is a therapeutic anti-inflammatory target. CD11b (alpha M integrin) complexes with CD18 (beta 2 integrin) to form complement receptor type 3 (CR3) heterodimer. Treatment with either monoclonal antibody NIMP-R10 or 5C6, both directed against CD11b resulted in decreased clearance of listeria in the liver and spleen with increased mortality (Conlan and North 1992; Burleson and Burleson 2006). Both neutrophils and monocytes were decreased and mice were unable to control the infectious intracellular bacterial disease. Treatment of mice with a surrogate biological mAb designated NIMP-R10, directed against the CD11b polypeptide of the CD18/CD11b heterodimer, exacerbated listeriosis by preventing myelomonocytic cells from focusing at sites of infected hepatocytes in the liver. Under these conditions, an otherwise sub-lethal listeria inoculum grew unrestricted

within hepatocytes and caused death within 3 days (Conlan and North 1992). The results obtained with NIMP-R10 are similar to those reported with a different anti-CD11b mAb (5C6) (Rosen et al. 1989; Conlan and North 1991). The *Listeria monocytogenes* host resistance model is thus an important tool in evaluating the potential for disease enhancement and increased susceptibility to opportunistic infections in therapeutics or chemicals that interfere with bacterial clearance mechanisms.

15.3.1.2 Evaluation of Anti-Inflammatory Therapeutics

The *Streptococcus pneumoniae* pulmonary host resistance model in Lewis rats has been used to assess the effects of anti-inflammatory agents on innate immunity (Komocsar et al. 2007). While anti-inflammatory drugs are important to human health, it is known that these drugs are immunosuppressive with respect to innate immunity. The *S. pneumoniae* model is capable of measuring the suppression of the innate immune response following administration of anti-inflammatory test articles. The ability to rank order the severity of innate immune suppression with multiple test articles within the same study enhances the utility of this model for screening potential drug candidates.

15.3.1.3 Evaluation of Therapeutics Targeting TNF-α

The *S. pneumoniae* host resistance model is also valuable for evaluating the importance of macrophage cytokines on bacterial host resistance and for evaluating therapeutic agents that target TNF-α. In addition, this host resistance assay is useful in selecting a lead compound among compounds with equivalent therapeutic efficacy based on immunosuppression.

Human biological therapeutics targeting inhibition of TNF-α have been used to treat inflammatory autoimmune diseases such as rheumatoid arthritis, psoriasis, and Crohn's disease. Decreased TNF-α as a result of treatment with anti-TNF-α has an effect on several biomarkers of infection. Studies have reported that treatment of mice with a mAb to TNF-α results in altered levels of TNF-α in the lungs and serum, decreased neutrophils and increased numbers of bacteria (impaired bacterial clearance) with decreased survival in mice infected intranasally with *S. pneumoniae* (van der Poll et al. 1997; Takashima et al. 1997; Benton et al. 1998; O'Brien et al. 1999). The streptococcal pulmonary host resistance model is thus an important means to assess the functional immunological capacity of macrophages and neutrophils as well as macrophage cytokines.

15.3.1.4 Marginal Zone B (MZB) Cell Evaluation

Bacteria encapsulated with a polysaccharide capsule, such as *S. pneumoniae* or *Hemophilus influenzae*, are blood-borne pathogens that present a unique challenge to the immune system (Pillai et al. 2005). Capsular polysaccharide antigens are

thymus-independent type 2 antigens (TI-2) (Mond et al. 1995) and stimulate the TIAR, thus effective immune responses are dependent on the presence of a functional marginal zone (Amlot et al. 1985; Harms et al. 1996; Guinamard et al. 2000). The MZB cell model in mice or rats measures bacterial clearance, hematology, cytokine production, and antibody production at selected time points over a 14 day period after intravenous infection to create a blood-borne infection. MZB cells in both humans and rodents are considered a critical host defense mechanism directed against encapsulated blood-borne pathogenic microorganisms. Immunotoxicity directed against MZB cells not only decreases protection against blood-borne pathogens, but also results in a depletion of immunological memory. In summary, the TIAR is decreased or ablated as a result of MZB cell immunotoxicity (F.G. Burleson 2008).

Histopathology can detect defects in the splenic marginal zone and immunophenotyping markers can be included to detect alteration in the number of MZB cells. Should an effect on MZB cells be observed, the pharmaceutical agent may be evaluated in the *S. pneumoniae* systemic MZB host resistance model for encapsulated bacteria. The *S. pneumoniae* marginal zone B cell model has been characterized in mice and Sprague Dawley rats with a systemic blood-borne infection by intravenous inoculation. Bacteria are quantified by determining the number of CFU in the spleen, liver, lungs, and blood over a 2 week period. Cytokines, hematology, immunophenotyping, and anti-streptococcal antibody (TIAR) are also quantified in this model (Burleson and Burleson 2006).

15.3.2 Viral Reactivation Host Resistance Assay

Evaluating the potential for latent viral reactivation is critical, as reactivation of latent virus may result in a fatal disease such as progressive multifocal leukoencephalopathy (PML).

There are three major subfamilies of herpesviruses (alpha, beta and gamma), each containing strains susceptible to latent viral reactivation. These include the alpha herpesviruses family of herpes simplex virus-1 (HSV-1), HSV-2 and varicella-zoster virus (VZV); the beta herpesviruses that include human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV); and the gamma herpesviruses that include Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and murine gammaherpesvirus-68 (MHV-68). Viruses capable of latent virus reactivation also include BK virus and JC virus. CMV (cytomegalovirus), EBV (Epstein-Barr Virus) and HSV (Herpes Simplex Virus) belong to the Herpesviridae virus family while BK virus and JC virus belong to the Papovaviridae virus family. While these viruses may belong to different families and subfamilies, there are many similarities between the viruses capable of causing latent/reactivated viral disease. All these viruses have double stranded DNA (the human polyoma viruses are circular); are ubiquitous in the human population; cause mild primary infections followed by a latent viral infection; and immunosuppression, especially a suppressed CMI results in reactivation of latent viral infection (G.R. Burleson 2008).

15.3.2.1 Murine Cytomegalovirus (MCMV) Latent Viral Model

The MCMV latent viral host resistance model can be used to evaluate immunotoxicity (Selgrade and Daniels 1995) or efficacy of antiviral therapies, as well as assess reactivation of latent viral disease due to immunosuppression (G.R. Burleson 2008). The MCMV reactivation model is useful when evaluating a pharmaceutical agent to determine if suppression of CMI or HMI results in reactivation of latent virus.

15.3.2.2 Murine Gamma Herpesvirus 68 (MVH-68) Latent Viral Reactivation Model

MHV-68 is a model for EBV, and both MHV-68 and EBV are lymphotropic, establish latency after acute lytic infection, and are associated with the development of lymphomas. A viral reactivation animal host resistance model with MHV-68 would allow the evaluation of immunosuppressive therapeutics for their ability to reactivate latent virus as measured by qPCR gene expression, plaque assay, and/or development of lymphomas (Mikkelsen et al. 2014; Aligo, Walker, et al. 2015; Aligo, Brosnan, et al. 2015).

15.3.3 Fungal Host Resistance

Candida albicans is a well-characterized fungal host resistance model in mice capable of evaluating complex interaction of innate and adaptive immunity, as both are important for recovery from Candidiasis infections, as well as evaluating immunotoxicity against fungal infections or efficacy of antifungal therapeutics. (Herzyk et al. 2001; Burleson and Burleson 2006). Ashman et al. and Netea et al. have reported on the immunological functions important for clearance of *Candida albicans* infections (Ashman et al. 2004; Netea et al. 2015). Both neutrophils and macrophages represent the first line of defense, while CMI with T cells is also essential for recovery from Candida infections.

In this model, Candida is administered intravenously, and clearance is evaluated on Days 4, 7, 14, and 21. The *Candida albicans* host resistance model allows an assessment of total immunocompetence by evaluating clearance of the organism. Clearance of a fungal infection requires an intact and functional immune system that incorporates a cascade of immune responses including: cytokine production, NK cell activity, macrophage activity, CTL activity and antibody production.

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Index

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