

## Chapter 2

# Cells of the Immune System

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**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.

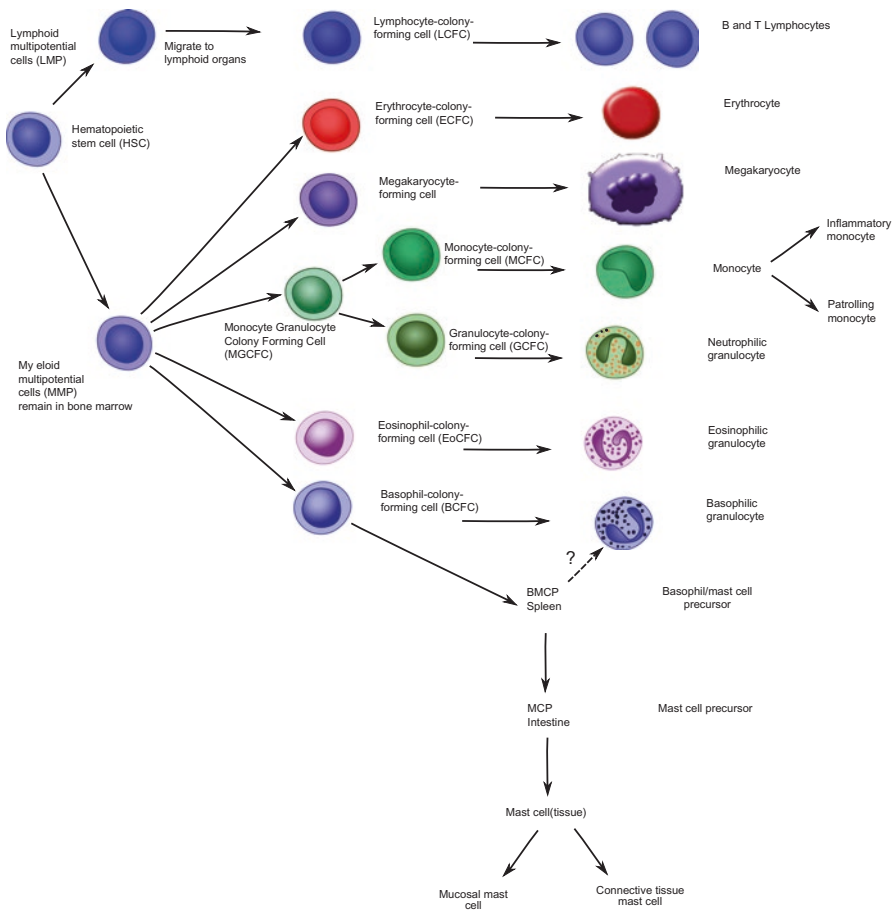
Within the multiple organs of the immune system and non-immune organs that have some level of immune functioning is a broad spectrum of cells that have primary or secondary roles in immune function. The goal of this chapter is to present basic immunobiological features of those cells and, to the extent 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  $\alpha\beta$ T 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  $\alpha\beta$  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 collectins, 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 ( $\text{Gr1}^+$ ) and patrolling ( $\text{Gr1}^-$ ) 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  $\text{TNF-}\alpha$ , 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<sup>+</sup> 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<sub>2</sub> (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<sup>+</sup> macrophages (den Haan and Martinez-Pomares, 2013). CD169<sup>high</sup> 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<sup>low</sup> 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<sup>low</sup> 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 (Perdiguerro 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 (Borreagaard 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  $\mu\text{m}$  diameter. Phagocytosis is triggered by neutrophil receptor recognition of polysaccharides on the surface of yeast cells, or binding of opsonized microorganisms to Fc $\gamma$  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-1 $\alpha$ ), CCL20 (MIP-3 $\alpha$ ), and CCL19 (MIP-3 $\beta$ ) (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- $\beta$  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- $\beta$  blockade results in a subpopulation of TANs that are hypersegmented, 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<sub>H</sub>17 cells produce mediators such as IL-17, the chemokine CXCL8 (also known as IL-8), interferon- $\gamma$ , tumor necrosis factor (TNF), and granulocyte-macrophage colony stimulating factor (GM-CSF), all of which serve to recruit, activate, and prolong the survival of neutrophils at sites of inflammation (Cua and Tato 2010). T<sub>H</sub>17 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- $\kappa$ B (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 dendritic 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 (Borreagaard 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 milieu 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 disassembly 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,  $\gamma\delta$  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 mediators 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 (Eggesten 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- $\alpha$ , 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 cytoplasmic 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 (Egsten 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- $\gamma$ , and TNF- $\alpha$  (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/EBP $\alpha$*  and *GATA2* (Iwasaki et al. 2006). *C/EBP $\alpha$*  and *MITF* are antagonistic transcription factors that silence each other, with *C/EBP $\alpha$*  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/EBP $\alpha$*  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  $\beta 2$  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 attachment 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 RceRI 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 known 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<sub>4</sub> 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<sub>H</sub>2 differentiation (Kumamoto et al. 2013; Otsuka et al. 2013; Tang et al. 2010) and present antigenic peptides to CD4<sup>+</sup> T cells (Otsuka et al. 2013). Basophils are also involved in the pathogenesis of the nephritis associated with lupus erythematosus (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 play key roles in airway and gastrointestinal diseases (Metcalf 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- $\alpha$  (C/EBP $\alpha$ ) 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/EBP $\alpha$  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  $\alpha 4\beta 7$  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  $\text{TNF}\alpha$ , 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 intricate 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. Mitochondria 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 mitochondria in a caspase-dependent manner, but granzyme B can also initiate caspase 3 processing and apoptosis in the absence of mitochondria (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 TCR $\alpha$  chain and a restricted assortment of TCR $\beta$  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  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) (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<sub>H</sub> cells, subdivides ILCs into three groups. Group 1 ILCs are those cells that produce IFN $\gamma$ , 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- $\alpha$  (ROR $\alpha$ ) for their development and function. Group 2 ILCs includes cells that have been previously described as natural helper cells, nuocytes and I<sub>H</sub>2 cells. Group 3 ILCs produce IL-17 and/or IL-22, and depend on the transcription factor ROR $\gamma$ t for their development and function. The prototypical ILC Group 3 cells are the LT $\alpha$ i 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 CD8 $\alpha$  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- $\alpha\beta$  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<sup>+</sup> and CD8<sup>+</sup> 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  $\alpha\beta$  versus  $\gamma\delta$  T cells, and differentiation into CD4<sup>+</sup> or CD8<sup>+</sup> 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<sup>+</sup> T cells requires binding of the DLL1 ligand on APC by Notch1 or Notch2, which leads to expression of multiple T<sub>C</sub> signaling and effector molecules (*Eomes* > Eomes transcription factor; *Gzmb* > serine protease granzyme B; *Ifng* > interferon- $\gamma$ ; and *Prfl* > perforin 1) (Cho et al. 2009; Maekawa et al. 2003). In naïve CD4<sup>+</sup> T cells, ligation of DLL1 and DLL4 activate Notch signalling and transcription of *Tbx21*, which encodes T-bet (T<sub>H</sub>1 cell transcriptional regulator) (Maekawa et al. 2003; Minter et al. 2005). During development of T<sub>H</sub>2 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<sub>H</sub>17 and T<sub>H</sub>9 subsets by promoting the expression of *Rorc* (encoding transcription factor Ror $\gamma$ t) 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<sup>+</sup> CD8<sup>+</sup> (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  $50 \times 10^6$  cells. The response of the naïve T cell is remarkably rapid and robust. A naïve CD8<sup>+</sup> 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  $\gamma\delta$  T cells, as opposed to the predominant  $\alpha\beta$  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 through the double-negative (DN)1 and DN2 stages, and reach a developmental checkpoint at the DN3 stage (CD4<sup>−</sup> CD8<sup>−</sup> CD25<sup>+</sup> CD44<sup>−</sup> cells) (Pearse 1989; Shinkai 1992). Only cells that succeed in TCR  $\beta$  chain gene rearrangement, known as the  $\beta$ -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 Freuden-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 TCR $\beta$  and pre-TCR $\alpha$  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 TCR $\alpha\beta$  (von Boehmer and Fehling 1997; Irving et al. 1998; Ciofani and Zúñiga-Pflücker 2005). Expression of

the TCR $\alpha\beta$  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 TCR $\alpha\beta$ , 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 as 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 (Boldizar 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 (Eggle 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<sup>+</sup> and CD3<sup>-</sup> subsets (Bluestone et al. 1987). In addition to the conventional population of  $\alpha\beta$  T cells, the CD3<sup>+</sup> thymocyte population contains CD3<sup>+</sup>  $\gamma\delta$  (Havran and Allison 1988) and CD3<sup>dim</sup> TCR $\alpha\beta$ <sup>+</sup> NK1.1<sup>+</sup> 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  $\alpha\beta$  heterodimers or  $\gamma\delta$  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 ( $\alpha$  and  $\gamma$  chains) or V, D and J chains ( $\beta$  and  $\delta$  chains) (Jenkins 2013). Each T cell typically expresses only a single type of  $\alpha/\gamma$  or  $\beta/\delta$  chain. The TCR complex also includes CD3 $\gamma$ ,  $\delta$ , and  $\epsilon$  as well as TCR $\zeta$  (or CD3 $\zeta$ ). 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 (CD8 $\alpha\alpha$ ) or heterodimeric (CD8 $\alpha\beta$ ).

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- $\kappa$ B 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<sup>+</sup> T cells respond to antigenic stimulation primarily by increased expression of IL-2, a growth factor. By contrast, CD8<sup>+</sup> 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 IFN $\gamma$ . In addition to the basic CD4<sup>+</sup> and CD8<sup>+</sup> subdivision, there are multiple T cell subsets such as T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, 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<sub>H</sub>1 function, GATA-3 promotes T<sub>H</sub>2 function, PLZF promotes NKT-cell function, ROR $\gamma$ T promotes T<sub>H</sub>17 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  $7 \times 10^7$  or  $3 \times 10^{11}$  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<sup>+</sup> 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- $\beta$ , or IL-6 and IL-21 become IFN- $\gamma$ -producing T<sub>H</sub>1 cells, IL-4-producing T<sub>H</sub>2 cells, IL-17-producing T<sub>H</sub>17 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<sup>+</sup> helper T cells, CD8<sup>+</sup> T cells require costimulatory signals from innate immune cells to reach their full effectiveness as cytotoxic T lymphocytes (CTLs). Naïve CD8<sup>+</sup> 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<sup>+</sup> T<sub>H</sub> cells complete with CD40-CD40L interaction, produce the requisite third signals that allow CD8<sup>+</sup> T cells to become functional CTLs.

The preponderance of T cell functions are associated with T cells that have  $\alpha$  and  $\beta$  TCR chains, but there is an additional population of T cells that have  $\gamma$  and  $\delta$  TCR chains. In some ways  $\gamma\delta$  T cells function as innate immune cells or non-conventional T cells, with several innate-like features that allow  $\gamma\delta$  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  $\gamma\delta$  T cell population arises early in ontogeny (GD 14 to GD 18 in mice), prior to development of the conventional

$\alpha\beta$  T cell population (Tonegawa et al. 1989; Raulet 1989). The  $\gamma\delta$  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  $\gamma\delta$  T cells in epithelial surfaces such as skin and mucosa of the gastrointestinal, respiratory and reproductive systems, allow  $\gamma\delta$  T cells to serve as an effective early response team.

The Fas/FasL pathway and the perforin-granzyme system are utilized by  $\gamma\delta$  T cells to kill infected, activated or transformed cells (Qin et al. 2009; Dieli et al. 2001). Products of  $\gamma\delta$  T cells include directly bacteriostatic or lytic molecules such as granulysin and defensins (Dieli et al. 2001; Dudal et al. 2006). The  $\gamma\delta$  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 IFN $\gamma$ ), 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,  $\gamma\delta$  T cells also produce down-regulatory cytokines such as TGF $\beta$  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  $\alpha\beta$  T cells restricted to the MHC class I-like CD1d molecule, and mucosa-associated invariant T (MAIT) cells, which are  $\alpha\beta$  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  $1 \times 10^7$  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- $\gamma$  (Cham et al. 2008). The above analysis indicates that CTLs contain approximately  $1 \times 10^7$  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-2R $\alpha$  are present in CTLs in approximately twofold abundance over those for IL-2R $\gamma$ , but proteomic analysis indicates there are 50 copies of IL-2R $\alpha$  for each molecule of IL-2R $\gamma$  (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<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> cells that constitute 5–10% of the total CD4<sup>+</sup> T cell population. They are characterized by the critical expression of Foxp3, a transcription factor that represses the expression of the T<sub>H</sub>1, T<sub>H</sub>2 or T<sub>H</sub>17 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<sup>+</sup> 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<sup>+</sup> CD4<sup>+</sup> 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  $\gamma$ t (ROR $\gamma$ t) 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.

### **$\gamma\delta$ 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  $\alpha\beta$  T cell populations that participate in this reverse cross-talk include cells that are restricted to the non-polymorphic MHC class-I-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  $\gamma\delta$  T cells, also participate in the reverse cross-talk by recognition of conserved non-peptide antigens that are up-regulated by stressed cells. The  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells allows the subpopulations of  $\gamma\delta$  T cells to develop immunological responsiveness to the pathogens that predominate 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 perforin and granzymes (Strid et al. 2008; Dieli et al. 2001). The  $\gamma\delta$  T cells also release a spectrum of signaling and effector molecules, including granulysin and defensins (Dieli et al. 2001; Dudal et al. 2006), TNF, IFN $\gamma$ , IL-17, IL-4, IL-5, IL-13, IL-10 and TGF $\beta$  (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  $\gamma\delta$  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  $\gamma\delta$  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  $1\text{--}2 \times 10^6$  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<sup>+</sup> VCAM1<sup>+</sup> 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 micro-environment 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<sup>+</sup> 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<sup>-</sup>. 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, glycoproteins, 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 pre-determined 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 lobar 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 NF $\kappa$ B 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<sup>+</sup> 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<sup>+</sup> 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  $\gamma\delta$  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<sub>H</sub>1 versus T<sub>H</sub>2 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 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> subgroups. The CD8 $\alpha$ <sup>+</sup> subgroup is biased toward MHC class I antigen presentation, while the CD8 $\alpha$ <sup>-</sup> 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 I interferons. The mDC subset, which is subdivided into CD103<sup>+</sup> and CD103<sup>-</sup> 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 progenitors), 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 I 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<sup>+</sup> 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 complexes 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 ‘icosomes’ 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-Specific Intercellular adhesion molecule-Grabbing Nonintegrin) 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- $\gamma$  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<sup>+</sup> 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<sub>H</sub>1–T<sub>H</sub>2 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<sub>H</sub>1 versus T<sub>H</sub>2 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  $\alpha 7$  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<sup>+</sup> and CD8<sup>+</sup> 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 S1P/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 inflamed 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 inflamed 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- $\alpha$  (PDGFR $\alpha$ ), 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  $\alpha$ -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<sup>+</sup> 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 TGF $\beta$ 1 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 FDCs 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 (Rozenendaal 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 (Rozenendaal et al. 2009; Junt et al. 2007). MRCs have many features in common with the lymph node stromal organizer (LT $\alpha$ ) 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 CCR4 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 chemokine 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 inflamed 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 (Sapozhnikov et al. 2008; Pereira et al. 2009).

Bone marrow stromal cells are involved in the generation and maintenance of memory CD4<sup>+</sup> T cells and memory plasma cells. The exact anatomic site of the conversion of antigen-dependent signaling to antigen-independent memory CD4<sup>+</sup> 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<sup>+</sup> effector T<sub>H</sub> 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<sup>+</sup> T<sub>H</sub> cells (Tokoyoda et al. 2009b). It is probable that a similar situation exists for memory CD8<sup>+</sup> 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  $14\text{--}20 \times 10^6$  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  $^3\text{H}$ 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 Fc $\gamma$  receptor, principally Fc $\gamma$  receptor IIB2 (Fc $\gamma$ RIIB2) and Fc $\gamma$  receptor III (Fc $\gamma$ RIII) 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 (IFN $\gamma$ )-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<sup>+</sup> TCR<sup>int</sup>) 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<sub>2</sub>-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  $4.12 \text{ min}^{-1}$ , 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 (TNF $\alpha$ ), 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 $\times$ ) 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<sup>+</sup> 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 as 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**

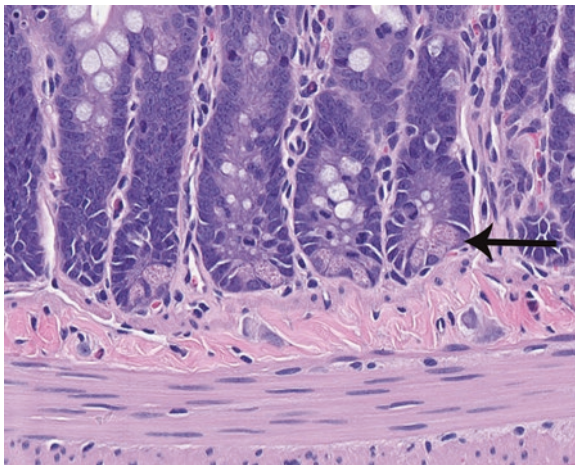
The innate mucosal barrier of the intestine consists of mucosal epithelial cells, mucins, IgA and antimicrobial effector molecules (Salzman 2010). In intact hosts, the intestinal microbiome is critical to host protection by forming a mucosal barrier



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 ( $\alpha$ ,  $\beta$ , and  $\theta$ ) of defensins that have been identified in mammals, only the  $\alpha$  and  $\beta$  classes have been identified in mice and humans (Salzman 2010). In humans,  $\alpha$  defensins are found in neutrophils and Paneth cells, while in mice the production of  $\alpha$  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, TNF $\alpha$ , RegII $\gamma$ , 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 $\times$  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 potent stimulators of adaptive immune responses, and are known to stimulate the expression of IL-17A by CD4<sup>+</sup> T cells (T<sub>H</sub>17 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 degranulation 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 polyethylene 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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